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International application number: PCT/NZ04/000317

International filing date: 07 December 2004 (07.12.2004)

Document type: Certified copy of priority document

Document details: Country/Office: AU  
Number: 2004901570  
Filing date: 24 March 2004 (24.03.2004)

Date of receipt at the International Bureau: 14 March 2005 (14.03.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)



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WITNESS my hand this  
Twenty-seventh day of January 2005

A handwritten signature in cursive script, appearing to read 'J. Peisker'.

JANENE PEISKER  
TEAM LEADER EXAMINATION  
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**AUSTRALIA**

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**PATENTS ACT 1990**  
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**PROVISIONAL SPECIFICATION**

***FOR THE INVENTION ENTITLED:-***

**"SET1 PROTEINS AND USES THEREFOR "**

The invention is described in the following statement:-

## **SET1 proteins and uses therefor**

### **FIELD**

The present invention relates to methods of use of SET1 (also known as SSL7) proteins or functional equivalents thereof. More particularly the invention relates to the use of  
5 SET1 proteins or functional equivalents thereof in procedures for identification and/or isolation of IgA and the serum complement factor C5.

### **BACKGROUND**

The gram-positive bacterium *Staphylococcus aureus* is a common human pathogen causing food poisoning, skin infections, abscesses, bacteraemia, pneumonia,  
10 endocarditis, osteomyelitis, toxic shock syndrome, staphylococcal scarlet fever and scalded skin syndrome. *S. aureus* produces a plethora of cell-surface and secreted proteins that bind host cell-surface receptors, extracellular matrix proteins, and soluble serum factors involved in both innate and adaptive immunity (1). Several studies (2) and the recent completion of several staphylococcal genomes has revealed that many of  
15 these virulence factors are clustered within 3 distinct pathogenicity islands SaPI<sub>n</sub>1, SaPI<sub>n</sub>2 and SaPI<sub>n</sub>3 (3, 4). One important class of genes within SaPI<sub>n</sub>1 and SaPI<sub>n</sub>3 are those that code for superantigens. These potent exotoxins target the antigen recognition receptors of the adaptive immune response - the Major Histocompatibility Complex class II (MHC-II) and T cell Receptor (TcR) and drive antigen independent T-cell  
20 activation and cytokine release (5) possibly in an attempt to prevent local inflammation and leukocyte recruitment to the site of infection through the production of T cell cytokines (4).

The staphylococcal exotoxin-like proteins (SETs) encoded by genes clustered within the staphylococcal pathogenicity island SaPI<sub>n</sub>2 are superantigen homologues (8).  
25 Comparative alignment of SETs and superantigen sequences indicate that they have evolved from the same ancestral gene (3) and are most similar to toxic shock syndrome toxin 1 (TSST-1) whose gene resides on SaPI<sub>n</sub>1. TSST-1 is a TcR V $\beta$ 2 binding superantigen produced by toxigenic strains of *S. aureus* associated with Toxic Shock Syndrome (9). Twenty-six members of the SET family have been identified (8) (3) (10),

although several appear to be allelic variants. For example SET1 from strain NCTC6571 (8), SET11 from N315 and Mu50 (3), and SET22 from MW2 (10) are probably the same protein. The 3-D structure of SET3 has been determined and displays the same OB-fold/ $\beta$ -grasp two-domain structure as the superantigen (11) with conservation of the central core region at the interface of the two domains. Yet the SETs are not superantigens. They do not bind MHC class II nor activate T cells. Those regions on SET3 corresponding to the MHC class II or TcR binding sites on superantigens are substantially altered (11). The function of SETs remains unknown although the presence of their genes on SaPin2 may indicate that they are part of the bacterial defense armamentarium (11) (8) (12). Notably an *set15*<sup>-</sup> mutant of *S. aureus* displayed a 30-fold reduction in bacterial persistence in a murine kidney abscess infection model.

SET proteins may also be known as staphylococcal superantigen-like proteins (or SSLs). There has been a move recently to use SSL when naming these proteins.

IgA is the predominant antibody located at mucosal surfaces and the second most predominant isotype in serum. In humans the IgA production of approximately 66mg/kg/day exceeds that of all other Ig classes combined (13). Mucosal IgA exists mostly as a dimer complexed with a J-chain and a secretory component (SC). Polymeric IgA binds to the polyIg receptor on the basolateral surface of the mucosal epithelium to be transcytosed through the epithelial layer and released into the mucosa as a covalent complex called secretory IgA (sIgA). The cleaved ectodomain of the pIgR remains bound as the secretory component (SC). Secretory IgA is unable to bind Fc $\alpha$ R and activate phagocytosis in the absence of an integrin co-factor Mac-1 (complement receptor 3 or CD11b) (17). (Reviewed in (15), (16)). Serum IgA on the other hand is predominantly monomeric and only binds avidly to its receptor Fc $\alpha$ RI (CD89) on formation of immune complexes. Binding and cross-linking of Fc $\alpha$ RI (CD89) by IgA immune complexes initiates phagocytosis by neutrophils, granulocytes and monocyte/macrophages (17). Monomeric serum IgA has been proposed to provide a second line of defense against microbes such as *S. aureus* via Fc $\alpha$ RI mediated phagocytosis (18, 19). Mutagenesis (20-22) and the recent crystal structure of an

IgA:Fc $\alpha$ R complex reveals that the Fc $\alpha$ R Ig-like D1 domain binds at the C $\alpha$ 2:C $\alpha$ 3 junction of the IgA H-chain(15).

Complement C5 is the central component in the terminal stage of the classical, alternative, and lectin mediated complement pathways. Complement C5 is ~189 kD and  
5 is synthesised as an intracellular single-chain precursor that is secreted as a two-chain glycoprotein consisting of a 75 kD N-terminal C5 $\beta$  fragment disulfide linked to a 115 kD C-terminal C5 $\alpha$  fragment ((23, 24)). The surface bound C5 convertases generated from either the classical, alternative or lectin pathway; cleave soluble C5 to generate two active fragments C5a and C5b. The potent anaphylatoxin C5a is a 74-residue N-  
10 terminus fragment cleaved from C5 $\alpha$  by C5 convertase. C5a binds a G-protein coupled receptor C5aR on the surface of myeloid cells to stimulate a range of pro-inflammatory and chemotactic actions such as oxidative burst, phagocytosis and leukocyte recruitment which all contribute to the defense against organisms such as *S. aureus* (25). The C5b fragment initiates assembly of the terminal complement components into the membrane  
15 attack complex (MAC) that forms a water permeable membrane channel leading to cell lysis.

Methods are known for the purification of immunoglobulins, including IgA. For example, purification may be achieved by methods including classical protein purification techniques, such as ion exchange and size exclusion chromatography, and  
20 by affinity purification using a number of bacterial derived proteins, such as Protein L. These techniques may suffer from a number of problems including being laborious, lacking specificity and can result in low yields and purity. For example, Protein L, derived from the bacterium *Peptostreptococcus magnus*, and which is used for purification of immunoglobulins, binds to a wide variety of immunoglobulins. This  
25 means that it may not be suitable for the purification of a single species of immunoglobulin from a heterogenous mix of immunoglobulins.

An example of a product which may allow for single step purification of IgA and IgE is the affinity purification product known as Kaptiv-ae<sup>TM</sup> (Genomics One International Inc, USA). This is a synthetic peptidomimetic compound that selectively binds IgA and IgE  
30 from several species.

Single step purification methods for complement C5 are not known. Current methods to purify complement C5 from human serum, for example, rely on multiple chromatographic steps such as ion exchange and size exclusion chromatography. These may often result in low yields of final product.

- 5 Accordingly, there may be considered a need to provide an alternative or improved method of isolating and identifying IgA and C5.

Bibliographic details of the publications referred to herein are collected at the end of the description.

#### **OBJECT**

- 10 It is an object of the present invention to provide an improved method of isolating and/or identifying IgA and/or C5 or at least to provide the public with a useful choice.

#### **STATEMENT OF INVENTION**

- 15 In accordance with the present invention it has been discovered that recombinant SET1 binds independently IgA and the serum complement factor C5. This may have significant application in methods of isolating or removing IgA and C5 from samples and also in identification and quantitation of IgA and C5 in samples for diagnostic purposes, for example.

In a first broad aspect of the invention there is provided a method of isolating IgA present in a sample, the method comprising at least the steps of:

- 20 Bringing SET1 or a functional equivalent thereof in contact with the sample for a period sufficient to allow SET1 to bind to IgA to form a complex;

Separating the complex.

Releasing IgA from the complex.

- 25 In a another broad aspect of the invention there is provided a method of isolating C5 present in a sample, the method comprising at least the steps of:

Bringing SET1 or a functional equivalent thereof in contact with the sample for a period sufficient to allow SET1 to bind to C5 to form a complex;

Separating the complex.

Releasing C5 from the complex.

- 5 In a preferred broad aspect of the present invention there is provided a method for isolating IgA from a sample, the method comprising at least the steps of:

Providing a matrix to which a SET1 protein or functional equivalent is bound;

Providing a sample;

- Bringing said matrix and said sample into contact for a period sufficient to allow SET1  
10 to bind to IgA present in the sample; and,

Releasing IgA from the matrix.

Preferably, the method further comprises the step of collecting the IgA released.

Preferably, the matrix is in the form of a column over which the sample is passed.

- Preferably the method further comprises the step of washing contaminants present in the  
15 sample from the matrix prior to release of IgA.

Preferably the matrix is Sepharose.

Preferably the sample is colostrum. More preferably the sample is serum.

Preferably the method further comprises the step of determining the quantity of IgA present in the sample.

- 20 Preferably, IgA is released from the matrix using a 100mM glycine buffer at pH 3.0.

In a preferred broad aspect of the present invention there is provided a method for isolating C5 from a sample, the method comprising at least the steps of:



Providing a matrix to which a SET1 protein or functional equivalent is bound;

Providing a sample;

Bringing said matrix and said sample into contact for a period sufficient to allow SET1 to bind to C5 present in the sample; and,

- 5   Releasing C5 from the matrix.

Preferably, the method further comprises the step of collecting the C5 released.

Preferably, the matrix is in the form of a column over which the sample is passed.

Preferably the method further comprises the step of washing contaminants present in the sample from the matrix prior to release of C5.

- 10   Preferably the matrix is Sepharose.

Preferably the sample is colostrum. More preferably the sample is serum.

Preferably the method further comprises the step of determining the quantity of C5 present in the sample.

Preferably, C5 is released low pH buffer such as 50mM acetate pH 3.5.

- 15

In another broad aspect the invention provides a method of detecting IgA and/or C5 in a sample, the method comprising at least the steps of:

Contacting a sample with SET1 or functional equivalent thereof for a period sufficient to allow SET1 or functional equivalent thereof to bind to IgA and/or C5; and,

- 20   Detecting bound SET1 or functional equivalent thereof.

Preferably, the method further includes the step of determining or quantifying the level of bound SET1.

Preferably, the method is conducted for the purpose of diagnosing IgA and/or C5 abnormalities in a subject.

Preferably the subject is a mammal, more preferably a human.

In a further broad aspect, the present invention provides a novel SET1 protein having the  
5 amino acid sequence SEQ ID NO: 6 or SEQ ID NO: 7.

In a related broad aspect, the invention provides a nucleic acid encoding a SET1 protein of SEQ ID NO:6 or SEQ ID NO:7.

Preferably, the nucleic acid sequence is that represented by SEQ ID NO:12 or SEQ ID NO:13.

10 The invention may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification of the application, individually or collectively, in any or all combinations of two or more of said parts, elements or features, and where specific integers are mentioned herein which have known  
15 equivalents in the art to which the invention relates, such known equivalents are deemed to be incorporated herein as if individually set forth.

## FIGURES

These and other aspects of the present invention, which should be considered in all its novel aspects, will become apparent from the following description, which is given by way of example only, with reference to the accompanying figures, in which:

- 20 **Figure 1.** Illustrates Purified recombinant proteins. 10  $\mu$ g of recombinant Fc $\alpha$ RI ectodomain produced in baculovirus (22) (lane 2) and 10  $\mu$ g of SET1 produced in *E. coli* (lane 3) were analyzed by SDS PAGE under reducing conditions. Samples of purified human polyclonal IgA were also run for size comparison.
- 25 **Figure 2.** Illustrates Serum proteins bound by SET1. A. Recombinant SET1 coupled to sepharose purified proteins from human serum (lane 3) but not from fetal calf

serum (lane 2) of PBS (lane 1) controls. **B.** Comparison of protein A (lane 1) and SET1 purified Ig (lane 2) showing that the heavy chain for protein A purified Ig is 50 kD while SSL7 was 60 kD. **C.** Proteins purified from human serum by either protein A (lane 1) or SET1 (lane 2) were analyzed by Western blotting using human isotype specific anti-sera. Only the anti-IgA anti-serum (panel 1) detected the SSL7 bound IgA heavy chain at 60 kD. Anti-IgD, IgG and IgM did not react to SET1 purified serum proteins.

**Figure 3.** Illustrates SET1 binds IgA and C5 from multiple sites, individuals and species. **A.** SET1 sepharose purified proteins from serum (10 $\mu$ l), breast milk (10  $\mu$ l), saliva (100  $\mu$ l), and tears (100  $\mu$ l). The arrow indicates the position of the secretory component (SC) associated with secretory IgA observed in breast milk, saliva and tear but absent from serum. The serum sample contains the additional C5 alpha and C5 beta chains that are absent in the breast milk, saliva and tears. **B.** SET1 purified proteins from the serum of 14 human volunteers. 10  $\mu$ l of serum was incubated with 10  $\mu$ l (50% v/v = 50  $\mu$ g) of SET1 sepharose. The positions of C5 alpha (110 kD) and C5 beta (70 kD) chains, visible in all 14 samples, are shown by the arrows. Note absence of IgA heavy chain in lane 12. The 50 kD protein that varied in concentration between individuals is IgG Heavy chain and is a result of naturally occurring anti-SET1 antibodies. **C.** SET1 binds IgA from human, chimpanzee, baboon, pig, (and possibly rat and horse) and C5 from human, chimpanzee, baboon, sheep, pig, rabbit and horse. SpA-sepharose (lanes A) or SET1-sepharose (= 50 $\mu$ g) (lanes B) was incubated with 10  $\mu$ l of serum from different species and eluted proteins examined by SDS PAGE.

**Figure 4.** Illustrates that biosensor analysis demonstrates nanomolar binding of SET1 to IgA and C5 through independent sites. Serum IgA was immobilized to a CM5 BIAcore biosensor chip using carbodiimide chemistry. **A)** Kinetic IgA binding analysis: SET1 was reacted with the immobilized layer at the indicated concentrations at a flow rate of 30 $\mu$ l/min. The data from 20 sensosograms (light grey curves) from 4 independent experiments are shown

globally fitted (black solid line), using the BIAevaluation version 2.1 software, to a model describing parallel binding to a heterogeneous ligand model. Two types of sites are indicated the first  $K_D1 = 1.0$  nM, the second  $K_D2 = 330$  nM,  $\chi^2 = 0.184$ ,  $n = 4$ . B) Equilibrium IgA binding analysis: SET1 was reacted with the immobilized layer at the indicated 12 concentrations at a flow rate of  $1\mu\text{l}/\text{min}$ . For each concentration of SET1 the equilibrium binding response to IgA was obtained after 120min. The data shown is one representative experiment. Analysis of 4 independent experiments indicated 2 types of sites; the first  $K_D1 = 1.1 \pm 0.2$  nM, the second  $K_D2 = 80 \pm 40$  nM. C) SET1 captured on IgA can simultaneously bind human C5: SET1 ( $1\mu\text{M}$ ) was reacted with the immobilized IgA layer ( $30\mu\text{l}$ , flow rate of  $10\mu\text{l}/\text{min}$ ) and a subsequent injection was made of either buffer (dashed line) or human C5 ( $0.3\mu\text{M}$ ,  $30\mu\text{l}$ ). D) Equilibrium C5 binding analysis: SET1 ( $1\mu\text{M}$ ) was reacted with the immobilized IgA layer ( $30\mu\text{l}$ , flow rate of  $10\mu\text{l}/\text{min}$ ) and a subsequent injection ( $122\mu\text{l}$ , flow rate of  $1\mu\text{l}/\text{min}$ ) was made of the indicated concentrations of human C5 ( $122\mu\text{l}$ ). For each concentration of C5 the equilibrium binding response to the SET1:IgA was obtained after 120min. The data shown is one representative experiment fitted to a single binding site. Analysis of 4 independent experiments indicated  $K_D = 18 \pm 1$  nM.

**Figure 5.** Illustrates Different alleles of SET1 bind IgA. **A.** Amino sequence alignment of known alleles of SET1. Two local clinical isolates (GL1 and GL10) were included in the alignment. Identity (\*), strong similarity (:), and weak similarity (.), at each amino acid position is indicated. **B.** The two alleles (GL1 and GL10) differ in 30/201 amino acids but bind equivalent amounts of IgA from the serum of three individuals ( $10\mu\text{l}$  used from each). IgG bound by SpA-sepharose is shown as a comparison to IgA. SET1-sepharose incubated without added serum indicates a small amount of no-covalently bound SET1 has dissociated from the sepharose matrix.

**Figure 6.** Illustrates SET1 inhibits IgA binding to granulocytes. IgA-FITC binding to human granulocytes (gray histogram) was added at 10  $\mu$ g. SET1 (0.1 – 100  $\mu$ g) (open histograms) was incubated with IgA-FITC prior to cell staining (upper panel). SEA was used as a negative control and does not inhibit cell staining by IgA-FITC (lower panel).

**Figure 7.** Illustrates SET1 inhibits the binding of IgA to rsFc $\alpha$ RI. **A.** No binding of 200 nM SET1 to immobilized Fc $\alpha$ RI was detected. **B.** Addition of increasing amounts of SET1 (0-150 nM) progressively prevented the binding of soluble IgA (100nM) to immobilized rsFc $\alpha$ RI. SET1 was incubated with IgA prior to binding the rsFc $\alpha$ RI immobilized chip surface. **C.** The 1min post-injection responses of IgA binding indicate the dose-dependent inhibition by SET1. At stoichiometric addition of SET1 (100nM) to the IgA binding (62 RU) to rsFc $\alpha$ RI was inhibited by 70%.

**Figure 8.** Illustrates SET1 blocks complement mediated hemolysis of human erythrocytes. **A.** Hemolysis by IB serum of human RBC was inhibited in a dose dependent fashion by recombinant SET1. Maximum inhibition by SET1 was achieved at 0.63  $\mu$ g for 25 $\mu$ l serum (equivalent to 1.0 nM SET1). **B.** Inhibition of JL serum. Maximum inhibition was achieved at 0.38  $\mu$ g SET1 equivalent to 0.26 nM). **C.** Addition of recombinant SET3 did not affect hemolysis. Each tube used 25  $\mu$ l of human serum. Curves represent the result of duplicate SETs.

**Figure 9.** Illustrates Recombinant SET1 increases survival of *E. coli* in plasma. Freshly isolated human plasma (250  $\mu$ l) was pre-incubated in duplicate with increasing concentrations of either recombinant SET1 or SET3 (control) prior to the addition of  $\sim 10^7$  *E. coli* K12 bacteria. Surviving bacteria were enumerated by plating on agar. Figures are expressed as a percentage of surviving colonies from the total number of bacteria added.

#### PREFERRED EMBODIMENT(S)

The following is a description of the present invention, including preferred embodiments thereof, given in general terms. The invention is further elucidated from the disclosure given under the section "Examples" which provides experimental data supporting the invention and specific examples thereof.

5 As mentioned herein before, there has been a recent move to standardise nomenclature used in relation to the SET proteins. Accordingly, they may now be known as SSL (staphylococcal superantigen-like) proteins. In line with this, the proteins SET1 (from strain NCTC6571), SET11 (from strains N315 and Mu50) and SET22 (from strain MW2), each of which may be referred to herein, may now be referred to as alleles of  
10 SET1 (SSL7). As described in Lina *et al*, the SSLs are now named in sequential order as they occur on the genome.

The inventors have surprisingly discovered that SET1 proteins bind independently the antibody IgA and the complement factor C5. This has significant application in the purification of both IgA and C5 from samples. An additional application is in the  
15 identification, including quantitation, of IgA and C5 in a sample.

The staphylococcal exotoxin-like proteins (SETs), encoded by genes clustered within the staphylococcal pathogenicity island SaPI<sub>n</sub>2 are superantigen homologues. The function or role of SETs is unknown but they do not possess any superantigen activity despite ancestral relatedness. The inventors show here that recombinant SET1 expressed in *E.*  
20 *coli* selectively binds with high affinity to human, primate and pig IgA. They have also shown that SET1 selectively binds serum complement factor C5 from a number of different species tested. SET1 blocked binding of IgA to cell surface bound Fc $\alpha$ RI (CD89) and stoichiometric addition of SET1 to IgA was sufficient to inhibit binding to purified recombinant Fc $\alpha$ R protein. SET1 was also a potent inhibitor of complement-mediated hemolysis. SET1 bound to both monomer and secretory forms of  
25 Immunoglobulin A, demonstrating that SET1 and the secretory chain (SC) binding sites on IgA do not overlap. These results indicate that SET1 is a soluble microbial defense factor with multiple activities that targets two important host innate protective mechanisms - Fc receptor mediated phagocytosis and complement mediated  
30 inflammatory responses to *S. aureus*.

A first embodiment of the invention relates to a method for isolating IgA and/or C5 from a sample using a SET1 protein or functional equivalent thereof. Generally, the method comprises at least the steps of: bringing SET1 or a functional equivalent thereof in contact with the sample for a period sufficient to allow SET1 to bind to IgA and/or C5 to form a complex; separating the complex; and, releasing IgA and/or C5 from the complex.

In a preferred form of this embodiment the method comprises at least the steps of: providing a matrix to which a SET1 protein or functional equivalent is bound; providing a sample; bringing said matrix and said sample into contact for a period sufficient to allow SET1 to bind to IgA and/or C5 present in the sample; and, releasing IgA from the matrix.

It should be understood that the terms "isolate", or "isolating" and the like indicates that IgA and/or C5 have been separated from at least one contaminating compound. It should be appreciated that 'isolated' does not reflect the extent to which IgA and/or C5 have been purified.

A SET1 protein of the invention may be exemplified by the following amino acid sequence which is provided on GenBank:

SET1 gi|6176432|gb|AAF05587.1|AF188835\_1 [Staphylococcus aureus subsp. NCTC8325] exotoxin 1 (set1) gene, set1-A allele, complete cds

MKLKTLAKATLALGLLTTGVITSEGQAVQAAEKQERVQHLHDIRDHLHRYYSSES  
FEYSNVSGKVENYNGSNVVRFPKQDQNHQLFLLGKDKEQYKEGLQGQNVFVV  
QELIDPNGRLSTVGGVTCKNNKTSETNTPLFVNKVNGEDLDASIDSLIQKEEISL  
KELDFKIRQQLVNNGLYKGTSKYGKIINLKDENEKVEIDLGDKLQFERMGDVL  
NSKDIRGISVTINQI [SEQ ID NO:1]

However, there is noted allelic variation in SET1 proteins. The inventors have shown that allelic variation does not alter the binding properties of SET1 to IgA or C5. Accordingly, the invention is applicable to allelic variants of SET1 proteins specifically exemplified herein (for example SET11 and SET 22 isolated from different strains of

*S.aureus*). Such allelic variants generally have at least approximately 80% similarity at the amino acid level and the ability to bind IgA and/or C5. The invention is also applicable to those proteins not considered allelic variants *per se* but which incorporate conservative (as is understood in the art) amino acid substitutions at one or more sites.

5 Additionally, the invention is applicable to proteins or peptides which represent addition/deletion mutants of the SET1 proteins specifically exemplified herein, or those in which non-naturally occurring or modified amino acids have been substituted for naturally occurring amino acids at one or more position. In accordance with the invention, such variants should retain the ability to bind to IgA and/or C5. These  
10 variants may be referred to herein as "functional equivalents".

The inventor's contemplate mutation of SET1 molecules to help facilitate selective binding of IgA or C5. This may aid in the methods of the invention. The binding by SET1 to IgA and to C5 has been found by the inventors to be independent of each other suggesting two individual and independent binding sites on the SET1 surface. Mutation  
15 of the IgA binding site for IgA will thus lead to SET1 that exclusively binds C5, or vice versa. These mutants are intended to be encompassed by the term "functional equivalent".

Identification of a binding site for IgA has localised it to a region on the surface of SET1 characterised by the external face called the oligosaccharide binding (OB) domain. All  
20 alleles of SET1 bind human IgA. The OB surface is one region that is conserved among alleles of SET1. The region predicted to encompass the IgA binding site on SET1 encompasses amino acids residue Asparagine 31, Lysine 35, Glutamic acid 37, Lysine 50, Aspartic acid 51. Accordingly, the inventors contemplate that mutation of one or more of these amino acids to alanine, for example, will generate SET1 capable of only  
25 binding to C5. Similarly, mutation of residues involved in the binding to C5 will generate a version of SET1 that only binds IgA.

Typically, a SET1 protein, and/or function equivalent thereof, will have an amino acid sequence having at least approximately 80% similarity to that of GenBank AAF05587.1 (SEQ ID NO: 1 above). Preferably, there will be at least approximately 85% amino acid  
30 similarity, more preferably at least approximately 90% similarity.



It should be noted that SET1 proteins and their functional equivalents may be of human or any other animal origin.

Specific examples of further SET1 proteins of use in the invention include:

[SEQ ID NO: 2] – provided on GenBank database

5 >SET1 gi|6176432|gb|AAF05587.1|AF188835\_1 Staphylococcus aureus exotoxin 1  
(set1) gene, set1-A allele, complete cds

MKLKTLAKATLALGLLTTGVITSEGQAVQAAEKQERVQHLHDIRDLHRYYSSES  
FEYSNVSGKVENYNGSNVVRFPKQDQNHQLFLLGKDKEQYKEGLQGQNVFVV  
QELIDPNGRLSTVGGVTKKNNKTSETNTPLFVNKVNGEDLDASIDSFLIQKEEISL  
10 KELDFKIRQQLVNNYGLYKGTSTYKGIINLKDENKVEIDLGDKLQFERMGDVL  
NSKDIRGISVTINQI

[SEQ ID NO: 3] – provided on DDBJ database

>SET11\_N315 gi|13700317|dbj|BAB41615.1| exotoxin 11 [Staphylococcus aureus  
subsp. aureus N315]

15 MKLKTLAKATLALGLLTTGVITSEGQAVHAAKEKQERVQHLYDIKDLYRYYSSSE  
SFEFSNISGKVENYNGSNVVRFNQEKQNHQLFLLGKDKDKYKKGLEGQNVFVV  
KELIDPNGRLSTVGGVTKKNNKSSETNTHLFVNKVYGGNLDASIDSFLINKEEVS  
LKELDFKIRKQLVEKYGLYKGTSTYKGITINLKDEKKEVIDLGDKLQFERMGDVL  
LNSKDIQNIQAVTINQI

20 [SEQ ID NO: 4] – provided on GenBank database

>SET11\_Mu50 gi|15923416|ref|NP\_370950.1| exotoxin 11 [Staphylococcus aureus  
subsp. aureus Mu50]

MKLKTLAKATLALGLLTTGVITSEGQAVHAAKEKQERVQHLYDIKDLYRYYSSSE  
SFEFSNISGKVENYNGSNVVRFNQEKQNHQLFLLGKDKDKYKKGLEGQNVFVV  
25 KELIDPNGRLSTVGGVTKKNNKSSETNTHLFVNKVYGGNLDASIDSFLINKEEVS

LKELDFKIRKQLVEKYGLYKGTTKYGKITINLKDEKKEVIDLGDKLQFERMGDV  
LNSKDIQNIAVTINQI

[SEQ ID NO: 5] – provided on GenBank database

>SET22\_MW2 gi|21282117|ref|NP\_645205.1| ORFID:MW0388~exotoxin homolog  
5 [Genomic island nu Sa alpha2] [Staphylococcus aureus subsp. aureus MW2]

MKLKTLAKATLALGLLTTGVITSEGQAVQAKEKQERVQHLYDIKDLHRYYSSE  
SFEFSNISGKVENYNGSNVVRFNQENQNHQLFLSGKDKDKYKEGLEGQNVFVV  
KELIDPNGRLSTVGGVTKKNNQSSETNTPLFIKKVYGGNLDASIESFLINKEEVSL  
KELDFKIRQHLVKNYGLYKGTTKYGKITFNLKDGEKQEIDLGDKLQFEHMGDV  
10 LNSKDIQNIAVTINQI

[SEQ ID NO: 6] – novel SET1 allelic variant isolated by inventors

>SET1 GL10 S.aureus Greenlane

KEKQERVQHLYDIKDLHRYYSSESFEFSNISGKVENYNGSNVVRFNQDGGQNHQ  
LFLLGEDKAKYKQGLEGQNVFVVKELIDPNGRLSTVGGVTKKNNQSSETNTPLF  
15 VKKVYGGNLDASIESFSINKEEVSLKELDFKIRQHLVKNYGLYKGTTKYGKITFN  
LKDGEKKEIDLGDKLQFEHMGDVLNSKDIQNIAVTLKQI

[SEQ ID NO: 7] – novel SET1 allelic variant isolated by inventors

>SET1 GL1 S.aureus Greenlane

KEKQERVQHLYDIKDLHRYYSSESFEFSNISGKVENYNGSNVVRFNQEKQNHQL  
20 FLLGEDKAKYKQGLQGQDVFVVKELIDPNGRLSTVGGVTKKNNQSSETNIHLL  
VNKLDGGNLDATNDSFLINKEEVSLKELDFKIRKQLVEKYGLYQGTSKYGKITII  
LNGGKKQEIDLGDKLQFERMGDVLNSKDINKIEVTLKQI

Nucleic acids encoding SET1 proteins and functional equivalents of the invention will  
be appreciated having regard to the information herein and the known degeneracy in the  
25 genetic code. However, exemplary nucleic acids include:

[SEQ ID NO: 8] – provided on GenBank database

>set1|AF188835|[Staphylococcus aureus subsp. aureus NCTC6571]

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1 atgaaattaa aaacgttagc taaagcaaca ttagcattag gtttattaac tactggtgtc
61 attacatcag aaggtaagc agtcaagcg |gcagaaaaac aagagagagt acaacattta
5 121 catgatatta gagatttaca tcgatactac tcatcagaaa gtttgaata tagtaatgtt
181 agtggttaagg ttgaaaacta caatggttct aacgttgtac gctttaaccc aaaagatcaa
241 aatcaccaat tattcttatt aggaaaagat aaagaacaat ataaagaagg tctacaaggc.
301 caaatgtct ttgtagtaca agaattaatt gatccaaacg gcagactatc tactgttggt
361 ggtgtaacga agaaaaacaa caaaacttct gaaactaata cacctttatt tgtaataaaa
10 421 gttaatggtg aagatttaga tgcattcaatt gactcattt taatccaaaa agaagaatc
481 tcattaaaag agcttgattt caaaattaga caacaattag ttaataatta cggattatat
541 aaaggtagat ctaaatacgg taaatcatt atcaattga aagacgaaaa taaagtagaa
601 attgatttag gtgataaatt acaattcgag cgcattggcg atgtgttgaa tagtaaagac
661 attagaggtg tatcagtcac tattaaccaa atttaa
```

15 [SEQ ID NO: 9] – provided on GenBank database

>exotoxin 11 |BAB41615.1 | [Staphylococcus aureus subsp. aureus N315].

```
1 atgaaattaa aaacgttagc taaagcaaca ttggcattag gcttattaac tactggtgtg
61 attacatcag aaggccaagc agtcacgca aaagaaaagc aagagagagt acaacattta
121 tatgatatta aagacttata tcgatactac tcatcagaaa gtttgaatt cagtaatatt
20 181 agtggttaagg ttgaaaacta taacggttct aacgttgtac gctttaacca agaaaaacaa
```

241 aatcaccaat tattcttatt aggaaaagat aaagataaat ataaaaaagg ccttgaaggc  
301 cagaatgtct ttgtggtaaa agaattaatt gatccaaacg gtagactatc tactgttggt  
361 ggtgtgacta agaaaaataa caaatcttct gaaactaata cacatttatt tgtaataaa  
421 gtgtatggcg gaaatttaga tgcataaatt gactcatttt taattaataa agaagaagtt  
5 481 tactgaaag aacttgattt caaaattaga aagcaattag ttgaaaaata tggtttatat  
541 aaaggtacga ctaaatacgg taagatcact atcaatttga aagacgagaa aaaggaagta  
601 attgatttag gtgataaact gcaattcgag cgcattgggtg atgtgttgaa tagtaaggat  
661 attcaaaata tagcagtgac tattaatcaa atttaa

[SEQ ID NO: 10] – provided on GenBank database

10 >exotoxin 11 | NP\_370950.1 | [Staphylococcus aureus subsp. aureus Mu50].

1 atgaaattaa aaacgtagc taaagcaaca ttggcattag gcttattaac tactggtgtg  
61 attacatcag aaggccaagc agtccacgca aaagaaaagc aagagagagt acaacattta  
121 tatgatatta aagacttata tcgatactac tcatacagaaa gttttgaatt cagtaatat  
181 agtggttaagg ttgaaaacta taacggttct aacgttgtag gctttaacca agaaaaacaa  
15 241 aatcaccaat tattcttatt aggaaaagat aaagataaat ataaaaaagg ccttgaaggc  
301 cagaatgtct ttgtggtaaa agaattaatt gatccaaacg gtagactatc tactgttggt  
361 ggtgtgacta agaaaaataa caaatcttct gaaactaata cacatttatt tgtaataaa  
421 gtgtatggcg gaaatttaga tgcataaatt gactcatttt taattaataa agaagaagtt  
481 tactgaaag aacttgattt caaaattaga aagcaattag ttgaaaaata tggtttatat  
20 541 aaaggtacga ctaaatacgg taagatcact atcaatttga aagacgagaa aaaggaagta

601 attgatttag gtgataaact gcaattcgag cgcatgggtg atgtgttgaa tagtaaggat

661 attcaaaata tagcagtgac tattaatcaa atttaa

[SEQ ID NO: 11] – provided on GenBank database

>exotoxin 22 | NC\_003923.1 | [Staphylococcus aureus subsp. aureus MW2]

5       1 gtgaaattaa aaacgttagc taaagcaaca ttggcattag gcttattaac tactggtgtg  
61 attacatcag aaggccaagc agtgcaagca |aaagaaaagc aagagagagt acaacattta  
121 tatgatatta aagacttaca tcgatactac tcatcagaaa gtttgaatt cagtaatatt  
181 agtggttaagg ttgaaaatta taacggttct aacgttgtac gctttaacca agaaaatcaa  
241 aatcaccaat tattcttate aggaaaagat aaagataaat ataaagaagg ccttgaaggc  
10       301 cagaatgtct ttgtgtaaa agaattaatt gatccaaacg gtagactatc tactgttggt  
361 ggtgtaacga agaaaaataa ccaatcttct gaaactaata cacctttatt tataaaaaaa  
421 gtgtatggcg gaaatttaga tgcataaatt gaatcatttt taattaataa agaagaagtt  
481 tctactgaaag aacttgattt caaaattaga caacatttag ttaaaaatta tggtttatat  
541 aaaggtacga ctaaatacgg taagatcact ttcaatttga aagatggaga aaagcaagaa  
15       601 attgatttag gtgataaatt gcaattcgag cacatgggag atgtgttgaa tagtaaggat  
661 attcaaaata tagcagtgac tattaatcaa atttaa

[SEQ ID NO: 12] – nucleic acid encoding novel SET1 protein [SEQ ID NO: 6]

>set1 | GL10 isolate

1 aaagaaaaac aggaacgtgt tcagcacctg tacgacatca aagacctgca  
20       51 ccgttactac tctccgaat ccttcgaatt ctccaacatc tccggtaaag

101 ttgaaaacta caacggttcc aacgttggtc gttcaacca ggaaaaacag  
151 aaccaccagc tgttcctgct gggatgaagac aaagctaaat acaaacaggg  
201 tctgcagggt caggacgttt tcgttgtaa agaactgatc gaccgaacg  
251 gtcgtctgtc caccgttggg ggtgttacca aaaaaaaca ccagtcctcc  
5 301 gaaaccaaca tccacctgct ggtaacaaa ctggacgggtg gtaacctgga  
351 cgctaccaac gactccttc tgatcaaca agaagaagt tccctgaaag  
401 aactggactt caaatccgt aaacagctgg ttgaaaaata cggctgtgac  
451 cagggtacct ccaatacgg taaatcacc atcatcctga acggtggtaa  
501 aaaacaggaa atcgacctgg gtgacaaact gcagtcgaa cgtatgggtg  
10 551 acgttctgaa ctcaaagac atcaacaaa tcgaagttac cctgaaacag  
601 atctaa

[SEQ ID NO: 13] – nucleic acid encoding novel SET1 protein [SEQ ID NO: 7]

>set1 | GL1 isolate

1 aaagaaaaac aggaacgtgt tcagacctg tacgacatca aagacctgca  
15 51 ccgttactac tctccgaat cctcgaatt ctcaacatc tccggtaaag  
101 ttgaaaacta caacggttcc aacgttggtc gttcaacca ggaaaaacag  
151 aaccaccagc tgttcctgct gggatgaagac aaagctaaat acaaacaggg  
201 tctgcagggt caggacgttt tcgttgtaa agaactgatc gaccgaacg  
251 gtcgtctgtc caccgttggg ggtgttacca aaaaaaaca ccagtcctcc  
20 301 gaaaccaaca tccacctgct ggtaacaaa ctggacgggtg gtaacctgga

351 cgctaccaac gactccttcc tgatcaacaa agaagaagtt tccctgaaag  
401 aactggactt caaaatccgt aaacagctgg ttgaaaaata cggctgttac  
451 cagggtacct ccaaatacgg taaaatcacc atcatcctga acggtggtaa  
501 aaaacaggaa atcgacctgg gtgacaaact gcagttcgaa cgtatgggtg  
5 551 acgttctgaa ctccaaagac atcaacaaaa tcgaagttac cctgaaacag  
601 atctaa

A SET1 protein or functional equivalent thereof may be made by any number of standard techniques known in the art, having regard to the amino acid and nucleic acid sequences identified herein before. By way of example, they may be isolated from  
10 natural sources such as purification from crude culture supernatants of strains of *Staphylococcus aureus* known to produce SET1, produced recombinantly having regard to the genetic code, or produced by chemical protein synthesis. An exemplary protocol for obtaining recombinant SET1 is described herein after under the heading "Examples"

In accordance with a preferred form of the invention, IgA and/or C5 are captured or  
15 isolated using affinity chromatography however a skilled person may readily recognise alternative techniques. Generally, an affinity column is prepared combining a SET1 protein or functional equivalent thereof, suitably immobilised on a support resin or matrix.

Any appropriate support resin as known in the art may be used. As it will be  
20 appreciated, choice of support resin may depend on the means by which SET1 is to be immobilised on it. Preferable support resins include Sepharose such as Sepharose 4B, cyanogen bromide-activated (CNBr-activated) Sepharose, AH-Sepharose 4B and CH-Sepharose 4B, activated CH-Sepharose 4B, Epoxy-activated Sepharose 6B, activated Thiol-Sepharose 4B, Thiopropyl-Sepharose 6B, covalently cross-linked Sepharose  
25 (sepharose Cl), and other resins such as nickel chelate resins, cellulose, polyacrylamide, dextran. Such resins may be purchased for example from Pharmacia Biotech. However, a skilled person may produce a resin themselves using methodology standard in the art

While the inventors have found that it is not necessary to use spacer molecules it should be appreciated that where desirable, and where one is not present on a resin as it may be purchased or manufactured, a spacer molecule may be added to the resin. Such spacer molecule may, in certain circumstances, facilitate the attachment of the ligand (SET1 or  
5 a functional equivalent thereof) to the resin, and also facilitate efficient chromatographic isolation of IgA and/or C5. Relevant spacer molecules will readily be appreciated by persons of ordinary skill in the art.

In addition, cross-linking of a support resin, or activation of resins may help facilitate chromatographic separation. Accordingly the invention encompasses this. While  
10 support resins which have been cross-linked and/or activated may be readily purchased (for example, Sepharose C1 or CNBr-activated Sepharose) skilled persons will readily appreciate methods for achieving such results themselves

It will be appreciated that SET1 proteins or their functional equivalents may be chemically modified where necessary and to facilitate attachment to the support resin  
15 while not destroying its ability to bind C5 and/or IgA.

Once the support resin is prepared and any modifications made to it and/or a SET1 protein or functional equivalent, the SET1 protein or functional equivalent may be immobilized on the support resin using standard methodology. By way of example, the protein and the resin may simply be mixed for a period of time (by way of example, 2  
20 hours) to allow for attachment of the protein to the resin. Subsequently, any active groups which may remain on the resin may be blocked by mixing with a buffer such as Tris at pH 8.0 for a period of time (for example 2 hours). The protein-resin may then be washed in an appropriate buffer, such as PBS, then suspended in an appropriate buffer and stored. In a preferred form of the invention where a Sepharose resin is used, the  
25 protein-resin is stored 1:1 in a PBS/0.025%  $\text{NaN}_3$  buffer at 4°C until desired to be used. Further preferred methodology is exemplified herein after under the heading "Examples".

SET1 or a functional equivalent thereof may be combined with the support resin in any desired ratio. In a preferred form of the invention using CNBr-activated Sepharose 4B,



SET1 is combined at 7mg of protein/ml of wet gel Sepharose. This typically results in a concentration of approximately 5mg protein/ml of Sepharose gel.

Once the affinity matrix or resin is prepared as mentioned herein before it may be formed into a column according to standard techniques readily known in the field. The  
5 column may then be washed with an appropriate buffer to prepare it for taking a sample. Such appropriate buffer includes for example PBS, or any other neutral pH buffer containing isotonic concentrations of NaCl. A sample may then be loaded onto the column and allowed to pass over the column. In this step, IgA and/or C5 present in a sample will adsorb to the column resin or matrix.

10 Appropriate "samples" from which IgA and/or C5 may be captured or isolated include milk, colostrums, serum, tears, saliva, secretions from the gut or any other secretions containing either IgA or complement C5. The sample may be of human, or other animal origin.

Once a sample has passed over the column it will generally be washed with an  
15 appropriate buffer to remove unbound or non-specific proteins or other compounds which may have been present in the original sample. Skilled persons will readily appreciate an appropriate buffer suitable for use. However, by way of example, a PBS/300mM NaCl buffer may be advantageously used.

IgA may be eluted from the column using 100mM glycine at pH 3.0. In a specific  
20 example, 10 column volumes of glycine is used. However, it will be appreciated that this may be varied. The IgA will generally be eluted into any buffer which is adapted to neutralize the elution buffer. The inventors have found that 1M Tris pH 8.0 is appropriate.

C5 may be eluted under similar conditions. The inventors note that C5 elutes at slightly  
25 higher pH. Accordingly, a low pH buffer such as 50mm acetate pH 3.5 may preferably be used for C5.

Following elution or release of IgA and/or C5 from the matrix or column, IgA and/or C5 may be further purified via any number of standard techniques. For example, eluates may be dialysed, or run through an affinity column of the invention again.

It should be appreciated that a chromatographic column in accordance with the invention  
5 may be gravity fed, or fed using positive or negative pressure. For example, FPLC and HPLC are applicable to a method of the invention.

Skilled persons will readily appreciate how to implement an HPLC system in relation to the present invention having regard to the information herein and standard methodology documented in the art.

10 Persons of ordinary skill in the art to which the invention relates will readily appreciate how scale up of bench top columns may be achieved. For example, one may increase volume of the affinity column consistent with the volume of sample to be processed. Commercial scale may be dependent on ensuring that the amount of coupled SET1 saturates the amount of ligand to be bound. Alternatively, large amounts of sample may  
15 be processed by repeated processing through a smaller SET1, affinity column. This has the advantage of not requiring so much SET1 but does rely on the reusability of the SET1 for recycling. The inventors have found that SET1 is very stable when used for purification of IgA and/or C5 and can be reused many times without loss of binding activity.

20 It should be appreciated that an affinity matrix can also be used in batch wise fashion where the solid matrix is added directly to the sample rather than passing the sample through a column. This offers simplicity, but may result in a less clean sample. Such techniques require a step to separate the matrix from the solution or sample. This is normally achieved by gravity sedimentation and decantation of the supernatant followed  
25 by washing, or separation of the affinity matrix by low pressure gravity or suction filtration.

The present invention has the advantage of providing a one step system for isolating IgA and/or C5. It should be appreciated that there may be instances where it is desirable to obtain a biological sample that is free from IgA and/or C5. The present invention will

allow for substantial removal of these molecules from a sample. The techniques described hereinbefore are suitable for achieving this end. It should be appreciated that where removal of IgA and/or C5 is the objective (as opposed to capture and purification of IgA and/or C5) it would not be necessary to release IgA and/or C5 from any SET1 (or  
5 functional equivalent) to which it is bound.

In addition, the inventors demonstration of binding between SET1 and IgA and/or C5, provides a means for detecting the presence, and quantifying the level, of these proteins in a sample. This has diagnostic significance in determining sero-conversion of mucosal immunity (IgA), complement competency (C5) and in detecting abnormalities in these  
10 proteins (for example deficiencies, or increased expression, in a subject).

In accordance with the above, the invention also provides methods for detecting the presence, and/or quantifying the level of IgA and/or C5 in a sample. The method will generally comprise the steps: contacting a sample with SET1 or functional equivalent thereof for a period sufficient to allow SET1 or functional equivalent thereof to bind to  
15 IgA and/or C5; and, detecting bound SET1 or functional equivalent thereof. The method preferably includes the further step of determining the level of bound SET1. Such a method is applicable to any sample which may contain IgA and/or C5. It is applicable to samples from humans and other animals.

Persons of skill in the art to which the invention relates will appreciate means by which  
20 these proteins can be detected and/or quantified. However, by way of example SET1 or a functional equivalent thereof may first be conjugated to peroxidase or alkaline phosphatase by chemical cross-linking using standard methods to produce a staining reagent. Samples can be added to ELISA plates and SET1 can be added at a fixed concentration to bind to any IgA or C5 bound to the plastic plate surface. Following  
25 washing, the amount of SET1 can be quantified by measuring the amount of peroxidase or alkaline phosphatase bound using established colorimetric methods that result in the production of a coloured compound which can be measured in an ELISA plate reader. The levels of IgA/C5 in the sample can be determined by comparing results against a standard curve of a known sample of either IgA or C5. An alternative example is to  
30 utilise a sandwich ELISA employing an anti-SET1 specific antibody. In this case the

anti-SET1 antibody is conjugated to either peroxidase or alkaline phosphatase. After the SET1 has incubated with the sample on the ELISA plate and excess washed away, the anti-SET1 antibody linked to the enzyme is incubated and washed clean.

## EXAMPLES

### 5 Methods

#### *SET1 protein expression and purification*

The *SET1* gene was amplified by PCR from the genomic DNA of two local clinical isolates of *Staphylococcus aureus* (designated GL1 and GL10) obtained from Greenlane Hospital (Auckland, New Zealand) using the primer sequences (Forward-BamH1)  
10 (Reverse-EcoR1) CGGGATCCAAAGAAAAGCAAGAGAGAG and  
GGAATTCTTAAATTTGTTTCAAAGTCAC. The PCR fragment was sub-cloned into the expression vector pET32a-3C and expressed in *E. coli* (AD494(DE3)pLysS) as an N-terminal thioredoxin fusion protein. The fusion protein was purified by Ni<sup>2+</sup> affinity chromatography, cleaved using 3C protease, and subjected to Ni<sup>2+</sup> affinity  
15 chromatography again to separate SET1 from thioredoxin as previously described (11). Purified SET1 was passed through a polymyxin B-agarose column to remove residual *E. coli* endotoxins (Sigma).

#### *Serum and secreted samples*

Primate sera were provided by kind donation from Auckland Zoo. Fresh sera from  
20 rabbit, cow, mice, pigs, horse, sheep and rat was obtained from the Auckland University Animal Research Unit. Human breast milk was provided as a kind donation from a lactating female laboratory technician. Saliva and tears were collected from laboratory individuals and stored under sterile conditions at 4°C.

#### *Production of recombinant FcαRI protein.*

25 Soluble FcαRI protein was produced by recombinant baculovirus infection of SF21 insect cells and purified by nickel affinity chromatography as previously described (26).

***Coupling of proteins to sepharose***

Protein in PBS, pH 8.0 was added to CNBr-activated Sepharose 4B (Pharmacia Biotech) at 5-7mg protein/ml of wet gel sepharose and mixed by rotation at room temperature for 2 hrs. Remaining active groups were blocked with the addition of Tris pH 8.0 to  
5 100mM and incubating for 2 hrs. The protein sepharose was washed a total of six times in PBS then suspended 1:1 in PBS/0.025% NaN<sub>3</sub> and stored at 4°C. Coupling typically resulted in concentrations of approximately 5 mg protein/ml of sepharose gel. Coupled gel was stored in PBS, 0.1% azide at 50% v/v at 4°C.

### ***SET1 binding assays***

10µl of SET1-sepharose (50% v/v) or protein A-sepharose (50% v/v) suspension was added to 10-50 µl serum or secretion and the volume made up to 500µl with lysis buffer (1% TX-100, 1% bovine hemoglobin, 140mM NaCl, 10mM Tris.Cl pH 8.0, 0.025% NaN<sub>3</sub>, 1mM PMSF, 1mM iodoacetamide) and incubated with rotation for 1 hour at 4°C. Sepharose without any protein coupled was used as a negative control to determine proteins bound independently of SET1. The samples were washed three times using wash buffer (1% TX-100, 0.1% SDS, 1% deoxycolate, 500mM NaCl, 10mM Tris.Cl pH 8.0), once with TSA (10mM Tris.Cl pH 8.0, 140mM NaCl, 0.025% NaN<sub>3</sub>), and once with 50mM Tris pH 6.8. Proteins were solubilised by boiling for 2-5 min in 10µl of SDS-PAGE sample buffer prior to running on a 10 or 12.5% SDS-PAGE gel.

### ***Western Blot Analysis***

Proteins bound by SET1-sepharose or protein A-sepharose were resolved by SDS-PAGE under reducing conditions then transferred to a nitrocellulose membrane using a BioRad transblot apparatus (BioRad Laboratories). The nitrocellulose membrane was incubated for 1 hour at room temperature in TTBS (140 mM NaCl, 10mM Tris.Cl pH 7.6, 0.1% Tween 20) containing 5% (w/v) nonfat milk powder to block non-specific binding. Goat anti-IgA, anti-IgD, anti-IgG, or anti-IgM (Kallestad) was incubated at 1 in 5000 dilution with the membrane fragments for 1 hour at room temperature in TTBS. After 3 x 15 minute washes in 5 ml TTBS the membrane was incubated with a 1 in 4000 dilution (in TTBS) of biotin labeled rabbit anti-goat IgG (Sigma) for 1 hour at room temperature followed by 3 x 5ml washes in TTBS. The membranes were then incubated with a 1 in 4000 dilution of peroxidase labeled avidin (Dako), washed 3 times in TTBS, and analyzed using an ECL Western blotting detection kit (Amersham Pharmacia Biotech) as per the manufacturer instructions.

### ***Flow cytometric analysis***

Polyclonal human IgA was purified from normal human serum by SET1-sepharose affinity chromatography). 1 mg of IgA was incubated with 1 mg fluorescein

isothiocyanate (FITC Sigma) for 1 hour at 4°C then separated from free FITC by chromatography on a 5.0ml column of Sephadex G25 (Pharmacia). Leukocytes were prepared from whole blood by first diluting whole blood 10-fold with 0.85% ammonium chloride solution to lyse erythrocytes and centrifuging remaining white cells at 2500 rpm x 10 min. Leukocytes were suspended at  $1 \times 10^7$  cells/ml in PBS/2% FCS. A 2-fold dilution series from 100µg to 6.25µg of SET1 or SEA was prepared in 100µl PBS/2% FCS. 10µg polyclonal IgA-FITC was added to each dilution of SET1 and incubated for 15 min at RT in the dark. 50µl of freshly prepared leukocytes ( $5 \times 10^5$  cells) was added and incubated for 15 min at RT in the dark. Cells were then fixed with the addition of 300µl 8% formaldehyde and incubating for 3 min at RT in the dark. Cells were washed twice with 1ml PBS/2% FCS, centrifuged at 400 x g for 1min, and resuspended in 0.5ml PBS/2% FCS. The cells were analyzed by flow cytometry (FACS Analyser, Becton and Dickinson) with selective gating on the Granulocyte population.

#### *Biosensor analysis*

SET1 binding to IgA: Purified human serum IgA (Calbiochem) was immobilized on a CM5 carboxydextran chip using carbodiimide chemistry to a level of ~300 response units (RU) as described previously (26) using a BIAcore2000™ (BIAcore). Purified SET1 in the concentration range 10 to 200 nM was injected over the chip at a flow rate of 30µl/min. The binding and dissociation kinetics were globally fitted using the BIAevaluation version 2.1 software. The analysis of the equilibrium binding used 120 min injections of SET1 in the concentration range 0.25 to 400 nM. The equilibrium binding response ( $R_{eq}$ ) at 120 min was fitted to the two-site binding model;

$$R_{eq} = B_1 \cdot A / (K_{D1} + A) + B_2 \cdot A / (K_{D2} + A)$$

Where  $B_1$ ,  $B_2$ ,  $K_{D1}$ ,  $K_{D2}$  are the respective binding capacities and dissociation constants of the two sites and  $A$  is the free analyte concentration.

Equilibrium C5 binding analysis: SET1 (1µM) was reacted with the immobilized IgA layer (30µl, flow rate of 10µl/min) and a subsequent injection (122µl, flow rate of 1µl/min) was made of human C5 (Sigma) in the concentration range 2 to 130 nM. For

each concentration of C5 the equilibrium binding response to the SET1:IgA was obtained after 120min as the response above that of the injection of buffer alone. The data was fitted to the single binding site model;  $R_{eq} = B \cdot A / (K_D + [A])$ .

The biosensor analysis of SET1 inhibition of IgA binding to Fc $\alpha$ RI used rsFc $\alpha$ RI produced by recombinant baculovirus infection of SF21 insect cells and purified by nickel affinity chromatography (26). Briefly rsFc $\alpha$ RI was immobilized on a CM5 carboxyldextran chip for high affinity 2:1 receptor capture of serum IgA described in (Wines et al 2001). Serum IgA (Calbiochem) at 100nM was injected (20 $\mu$ l, 10 $\mu$ l/min) as the analyte and subtraction from a chemically coupled flow cell used to correct for bulk refractive effects. The inhibition of IgA binding was observed by incubating 100nM IgA with 0–150 nM rSET-1, 30min 25°C, prior to injection on the BIAcore. A binding response report point 1min after the injection was used to analyze rSET1 inhibition.

#### ***Complement mediated hemolytic assay***

The assay to detect hemolysis via the alternative complement pathway mediated was followed according to protocols in Handbook of Immunology (27). Serum from normal human individuals was tested for spontaneous hemolytic activity towards allogeneic human red blood cells from individual JF in a standard alternative complement pathway dependent hemolytic assay. Two sera (IB and JL) were found to be lytic. Heat inactivation (56°C for 30 minutes) of sera or addition of ethylene diamine tetra acetic acid (EDTA) reduced lysis to background, while addition of ethylene glycol tetra-acetic acid (EGTA) to selectively remove Ca<sup>++</sup> essential to the classical pathway, had no affect indicating that lysis was via the alternate pathway. Fresh human erythrocytes were prepared by repeated washing in Gelatin/Veronal buffered saline (GVB = 50mM diethyl barbiturate pH7.4, 1% gelatin, 0.15M NaCl). GVB++ buffer is GVB with CaCl<sub>2</sub> and MgCl<sub>2</sub> added to 0.06 mM and 0.4 mM respectively. GVBE buffer is GVB with 0.4 mM MgCl<sub>2</sub> and 10 mM EGTA. Erythrocytes were standardized at 2 x 10<sup>8</sup> cells/ml in ice cold GVB++. Immediately prior to use, 50  $\mu$ l of serum diluted 1:2 with GVB++ buffer (25  $\mu$ l equivalent), was incubated for two hours at 37°C with varying concentrations of SET1 protein in duplicate in 12 x 75 mm borosilicate glass tubes. 100  $\mu$ l human



erythrocytes and 50 µl of GVB++ buffer were added and the tubes incubated for 1 hour at 37°C. For total hemolysis, 100 µl of water was added instead of GVB++. To provide a negative control, GVB++ buffer replaced the serum. After 1 hour, 1.2 ml of ice cold 0.15M NaCl was added, tubes were centrifuged at 1250 rpm and hemolysis was  
5 measured by the absorbance at 412nm of the supernatant.

#### ***Plasma bacteriolytic assay***

250 µl aliquots of fresh heparin treated human plasma were pre-incubated in duplicate for 1 hour at 37°C with varying concentrations of recombinant SET1 or SET3 in 5 ml borosilicate glass tubes. 10 µl (10<sup>7</sup> cfu) of a fresh mid-log phase (OD 0.25) culture of *E.*  
10 *coli* K12 (DH5α strain) which is sensitive to plasma cytolysis was added to each sample then incubated with intermittent mixing for 3 hours at 37°C. 50 µl (1/5<sup>th</sup>) was plated onto LB plates and cultured for 24hrs at 37°C. Surviving colonies were counted the following day. Results were expressed as a percentage of surviving colonies over the total number of bacteria cells added to the plasma.

#### 15 **Results**

##### ***SET1 binds IgA and complement C5 from human serum.***

Recombinant SET1 (Fig. 1, lane 3) and recombinant soluble FcαRI CD89 (Fig. 1, lane 2) were produced to a high level of purity from *E. coli* and baculovirus infected Sf21 cells respectively (26). Recombinant SET1 was incubated with normal human  
20 peripheral blood lymphocytes to test for superantigen activity using a standard 3-day PBL proliferation assay. No proliferative activity was observed over the entire range of SET1 concentrations used (0.1pg/ml – 10 µg/ml) confirming lack of superantigen activity (data not shown).

Recombinant SET1 was chemically coupled to sepharose to investigate serum proteins  
25 that might bind SET1 directly. Four serum proteins were routinely purified by SET1 from all human sera tested (except for one individual – see below). The two predominant polypeptides of ~60 kD and ~27 kD were observed under reducing conditions but under non-reducing SDS PAGE migrated as a single high MW protein

consistent with immunoglobulin heavy and light chain polypeptides (fig. 2a, lane 3). The identity of the polypeptides excised from the SDS PAGE gel was confirmed by MALDI-TOF mass spectrum fingerprinting (performed by the Australia Proteome Analysis Facility). The 60kD band was unequivocally identified as human IgA heavy chain while the 27kD band was confirmed as the Ig light chain. In addition to IgA H and L-chains, two other serum polypeptides with molecular weights of 110 kD and 75 kD were consistently purified by SET1 from all individual sera tested. These were less predominant than the IgA bands, but nevertheless were consistently observed at approximately the same level across all individuals tested. Unambiguous identification of these polypeptides was again made by MALDI-TOF mass spectrometry (Australian Proteome Analysis Facility) from bands excised from the SDS PAGE. All samples gave unequivocal matches to human complement component C5 which in serum is composed of two covalently linked chains; C5 $\alpha$  chain (115kD) and C5 $\beta$  chain (75kD) consistent with the sizes of the bands observed on SDS-PAGE.

Proteomic analysis identified IgA as the predominant species bound by SET1 in serum, but could not exclude other isotypes that might be present in lower abundance. SET1 purified Ig H-chain was compared by SDS PAGE to proteins purified by staphylococcal protein A sepharose (SpA-sepharose) that predominantly binds the IgG isotype but also weakly binds other isotypes in humans. The SpA purified IgG H-chain (~50kD) (fig. 2B lane 1) was sufficiently separated in size by SDS PAGE from IgA H-chain (~60kD) (fig. 2B lane 2) to show that very little IgG isotype was bound by SET1. To further confirm this, the SET1 purified proteins were reacted with isotype specific anti-sera by Western blot. Only the anti-IgA antiserum reacted with the protein purified by SET1. Anti-IgG, IgD and IgM were negative (Fig. 2C lane 2). In contrast, all antisera reacted with the SpA purified protein (Fig. 2C lane 1), but the anti-IgG sera was strongest consistent with cross-reactivity of each of the isotype specific anti-sera towards IgG heavy chain. This confirmed that SET1 was selective for the IgA isotype.

***SET1 binds both serum and secreted IgA from all individuals and displays differing species specificity.***

Secretions known to have high levels of IgA were tested for SET1 reactivity. To account for the different concentrations of secretory IgA in each fluid; 10 $\mu$ l of serum, 10 $\mu$ l human breast milk, 100 $\mu$ l of saliva and 100  $\mu$ l of tears were reacted with SET1 sepharose. SET1 selectively purified either the monomeric form of IgA from serum  
5 (along with the C5  $\alpha$  and  $\beta$ -chain polypeptides), or the dimeric form of IgA with an additional 90kD band. This 90kD polypeptide was only observed in the breast milk, saliva and tears samples and was consistent with secretory component (SC) found only in mucosal IgA (Figure 3A). The size of the secretory chain from breast milk was slightly lower than the SC from saliva or tears presumably a result of an alternative  
10 proteolytic cleavage in mammary tissue.

SET1 binding of serum IgA might conceivably be a result of a profound sero-conversion through prior immunization with SET1 producing *S. aureus*. If this were true, then the amount of IgA purified by SET1 from the same volume of sera would be expected to vary between individuals. 10  $\mu$ l aliquots of serum from 14 individuals were incubated  
15 with an equivalent, saturating amount of SET1 sepharose and examined by SDS PAGE. The amount of IgA bound by 13/14 normal volunteers was uniform and consistent suggesting that binding was not unlikely to be via idiotypic regions of the IgA protein. Further studies confirmed that a saturating amount of SET1 extracted all the IgA present in serum with little IgA remaining following a single pass over SET1 sepharose (data not  
20 shown). Some individuals (lane 2, 10, 11 and 14 for example) had an additional band at 50kD that was later confirmed as IgG H-chain (not shown) which provided an excellent contrast between the uniform reactivity towards IgA, and the inconsistent idiotypic reactivity of IgG resulting from variable sero-conversion to SET1. The exception was volunteer 12 (fig. 3B) who had no detectable SET1 reactive IgA and compensatory  
25 levels of anti-SET1 IgG. The absence of IgA in individual 12 was later confirmed to be a result of a genetic IgA deficiency.

Similar amounts of C5 were bound by SET1 from all sera although individuals 5 and 7 showed slightly lower reactivity that might reflect lower serum concentration of C5. C5 binding was clearly apparent in individual 12 confirming that SET1 binding to C5 was

independent of IgA binding. This was later confirmed by Biosensor binding analysis (see below).

Analyses were performed on sera from other species and compared to reactivity with staphylococcal protein A (SpA) bound IgG. SET1 strongly bound IgA from human, chimpanzee, baboon and pig sera and displayed either weak (horse and rat) or no visible binding to cow, sheep, mouse, rabbit, goat IgA. In contrast, SpA bound IgG from the serum from all species (fig. 3C). SET1 bound C5 from sheep, horse and the rabbit serum in the absence of IgA binding confirming that IgA and C5 bound to SET1 independently. The most interesting reactivity was observed in rabbit where SET1 was highly selective for C5 (Fig. 3C). While this experiment did not account for the varying levels of IgA and C5 in the different species sera, it nevertheless confirmed that SET1 had different species selectivity for IgA and C5 proteins.

***SET1 has nanomolar affinity for both human IgA and C5.***

Surface plasmon resonance (BIAcore biosensor) was used to establish the affinity of the SET1 binding to human IgA. Recombinant SET1 protein was reacted with immobilized serum IgA (Calbiochem) and global analysis of the kinetic data fitted well ( $\chi^2 = 0.184$ ) to a model of parallel binding to a heterogeneous ligand with  $K_{D1} = 1.0$  nM and for the second site  $K_{D2} = 330$  nM (fig 4A). The fit was poor to other models including a single site model. The random coupling of ligand by carbodiimide chemistry to a biosensor chip can result in some orientations of ligand unfavorable for interaction with the analyte. Thus the high affinity site,  $K_{D1} = 1.0$  nM, most probably reflected the IgA:SET1 interaction. Since serum IgA consists of both IgA1 and IgA2 subclasses this also may contribute to the heterogeneity of the binding behaviour observed. Equilibrium binding analysis used long injection times (120 min) and the plateau of SET1 binding fitted best, like the kinetic data, to a two site binding model yielding  $K_D$  values of  $1.1 \pm 0.2$  nM and  $80 \pm 40$  nM respectively for the high and low affinity sites (fig. 4B). Thus both the kinetic and the equilibrium analysis concur that SET1 displays nanomolar affinity for serum IgA. The binding to recombinant baculovirus produced IgA1-Fc region and to recombinant IgA2 (Gift of Drs Margaret Goodall and Roy Jefferies) confirmed that

SET1 binds to both IgA1 and IgA2 isotypes in the Fc region of the molecule (data not shown).

The interaction of SET1 with human C5 was also analyzed using the biosensor. SET1 protein was injected over the immobilized IgA and a subsequent injection of purified  
5 human C5 (Sigma) demonstrated C5 binding to the SET1 captured on IgA (fig 4C). This simultaneous binding of IgA and C5 to SET1 indicates these interactions occur through distinct and functionally separate sites. This separation of binding functions agrees the serum binding studies that showed C5 from some species (e.g. rabbit) showed binding to SET1 independent of binding to IgA (fig 3C) and volunteer 12 who's  
10 complement C5 bound to SET1 in the absence of serum IgA.

The affinity of the SET1 interaction with human C5 was ascertained using IgA to capture SET1, which was then reacted with C5 in the analyte. In this case kinetic analysis was not possible because of the dissociation of the captured SET1 from the IgA layer. However equilibrium analysis was possible with the assumption that the binding  
15 of C5 to SET1 did not affect the rate of dissociation of SET1 from the IgA layer. The data obtained fitted well to a single binding site, as often occurs with orientated presentation of ligands to analyte on the biosensor, with a  $K_D = 18 \pm 1$  nM (fig 4D).

***Allelic differences in SET1 do not alter IgA or C5 binding.***

Some SETs such as SET1 exhibit considerable allelic variation among strains of *S.*  
20 *aureus*. To date, 4 allelic versions of the *SET1* gene have been published (MW2, NCTC8325, N315, NCTC6571). The amino acid sequences of these published alleles vary by 16% (fig. 4A). Two further alleles of *SET1* were obtained from local clinical isolates called GL1 and GL10. These were cloned and sequenced and compared for their ability to bind human serum IgA. SET1-GL1 and SET1-GL10 differ by 30/201  
25 residues; a variation of 15% yet both bound similar amounts of human IgA from identical volumes of serum from three different people (fig. 5B). Thus the IgA binding was not significantly altered by the 15% allelic variation observed between the GL1 and GL10 alleles.

***SET1 blocks IgA binding to myeloid cells.***

The receptor for IgA is Fc $\alpha$ RI (CD89) and is expressed on myeloid cells such as neutrophils, granulocytes, eosinophils, monocytes and macrophages (28). Flow cytometric analysis (FACS) of granulocytes with FITC-serum polyclonal IgA was performed to determine whether SET1 blocked the binding of IgA to the surface of granulocytes. SET1 was incubated at serial concentrations with a fixed concentration of FITC-IgA (10  $\mu$ g) prior to incubation with freshly prepared human leukocytes. The superantigen, staphylococcal enterotoxin A (SEA), was used as a negative control. Dose dependent inhibition of IgA binding to cells was observed. 90% inhibition was achieved with a 10-fold (w/w) excess of SET1 (100  $\mu$ g) over FITC-IgA (10  $\mu$ g). This was equivalent to 70-fold molar excess. SEA had no inhibitory effect on FITC-IgA binding (Fig. 6). This confirmed that SET1 bound to a site on IgA overlapping the Fc $\alpha$ RI binding site in the C $\alpha$ 2:C $\alpha$ 3 domains of the IgA H-chain (17).

***SET1 blocks binding of IgA to purified Fc $\alpha$ RI***

To confirm SET1 inhibits of IgA binding to Fc $\alpha$ RI, biosensor studies were performed using the Fc $\alpha$ RI ectodomains expressed using recombinant baculovirus infected Sf21 insect cells. The rsFc $\alpha$ RI was immobilized at a level of 500RU on a BIAcore CM5 biosensor chip. No binding of SET1 (200nM) to the Fc $\alpha$ RI was observed (Fig. 7A). In the absence of SET1 binding of the 100nM serum IgA to the Fc $\alpha$ RI was observed (~220 RU) with kinetics as reported previously (fig. 6B). Prior addition of SET1 inhibited the binding of IgA to immobilized Fc $\alpha$ RI. An approximately equimolar concentration of SET1 (100nM) to IgA (100nM) reduced the receptor binding by 70% (62 RU, Figure 6c). Thus SET1 inhibits IgA binding to Fc $\alpha$ RI both in its cell surface form and as soluble proteins.

***SET1 inhibits complement-mediated lysis and plasma bactericidal activity.***

Serum from normal individuals was tested for naturally occurring complement lysis of allogeneic human RBC. Two sera IB and JL were found that readily lysed allogeneic RBC in a standard *in vitro* complement hemolytic assay in the presence of EGTA. IB

serum was significantly more hemolytic lytic (100% hemolysis at 2-fold dilution) than JL (40% hemolysis at 2-fold dilution). Hemolysis was negligible following serum heat inactivation (56°C for 30 minutes) or addition of EDTA but was unaffected by EGTA confirming the activation of the alternate complement pathway (not shown).

- 5 Addition of recombinant SET1 to the serum prior to the addition of erythrocytes had a profound inhibitory effect on both IB and JL serum initiated hemolysis (Fig. 8). Maximum inhibition by SET1 was obtained on IB serum at 1.0 nM (0.63 µg/25 µl serum) of SET1. JL serum was completely inhibited at 0.26 nM (0.16 µg/25 µl serum) of SET1. Interestingly, the approximately 3-fold lower amount of SET1 was required to  
10 inhibit JL lysis correlated with the 3-fold lower lytic activity of JL serum. As a control, purified recombinant SEA that binds neither serum IgA nor complement C5, displayed no inhibition even at the highest concentration tested.

- SET1 was tested for its ability to provide protection to a bacterium that was sensitive to plasma bactericidal killing. Recombinant SET1 was incubated with fresh human plasma  
15 over a range of concentrations prior to the addition of  $\sim 10^7$  *E. coli* K12 bacteria, a bacterium which does not survive in human blood. No surviving cells were detected after 3hr incubation of  $10^7$  bacteria with 250 µl plasma but addition of recombinant SET1 at concentrations above 0.3 nM (7 µg/ml) consistently inhibited plasma anti-microbial activity and increased the number of surviving *E. coli* cells to a maximum  
20 level of around 0.03% at 1.72 nM (40 µg/ml) (fig. 9). The control recombinant SET3, had no effect even at the highest concentration used (3.44 nM = 80 µg/ml). IgA is commonly found in serum at approximately 12 nM (2 mg/ml) and C5 at approximately 0.4 nM (75 µg/ml). Based on simple concentration equivalence, inhibition of C5 by SET1 was the most likely basis for increased bacterial survival.

## 25 Discussion

Herein describes the first reported function(s) for a member of the eleven gene cluster of SETs (or SSLs) located on the staphylococcal pathogenicity island, SaPI<sub>n</sub>2 ((3), (10)). SET1 (or SSL7) selectively bound both purified IgA ( $K_D = 1 \pm 0.2$  nM) and complement C5 ( $K_D = 18 \pm 1$  nM) and inhibited complement and IgA:FcαRI binding - two important

components of the host protective immune mechanisms against *S. aureus*. SET1 also inhibited the bactericidal activity of plasma against a sensitive organism (in this case *E. coli*) at concentrations consistent with the approximate serum concentration of complement C5. The structural homology between SETs and the superantigens argues a close evolutionary relationship between these protein families, but this study reveals that the prototypical SET family member, SET1, has unique functions not shared with the superantigen family. These functions, leukocyte IgA receptor inhibition and complement inhibition, mediated through binding IgA and C5 respectively, reflect specialization of this superantigen-like class in microbial defense. Superantigens have variable surfaces to provide a range of binding options to both MHC class II molecules and T cell Receptors (5). Reminiscent of the conserved structural module but diverse functions of the immunoglobulin super-fold, these SET1 functions further diversify the binding possibilities for the two domain  $\beta$ -grasp OB-fold shared by the SET and superantigen families.

Two SET1 alleles that differ in 30/201 residues both bound IgA indicating that the variation did not disrupt this activity at least.

SET1 bound with high affinity to IgA and inhibited IgA:Fc $\alpha$ RI complex formation most probably through competition for binding at the IgA-Fc C $\alpha$ 2-C $\alpha$ 3 interface where the first ectodomain of Fc $\alpha$ RI binds (17, 20-22, 26). SET1 inhibition of IgA binding to Fc $\alpha$ RI was seen in a biosensor assay configured for high affinity bivalent capture of IgA (17, 26) and 70% inhibition was achieved by 1:1 addition of 100nM SET1 with 100nM IgA. The high affinity ( $K_D \sim 1$  nM) binding of SET1 to each IgA would result in about 3/4s of the IgA molecules having one or both heavy chains occupied by SET1 and so unavailable for bivalent capture by immobilized rsFc $\alpha$ RI. Moreover, SET1 bound equally sIgA and monomeric IgA from serum, indicating that the SET1 could affect immune evasion both in humoral and mucosal environments. Competition of SET1 and rsFc $\alpha$ RI for binding to IgA suggests that the most likely scenario is two SET1 binding sites per IgA-Fc. Furthermore SET1 binding of secretory IgA suggests that either the SET1 and SC binding site are completely separate, or the single SC in the sIgA incompletely occupies the SET1 binding sites.



SET1 is the first example of a staphylococcal immunoglobulin binding protein that selectively targets IgA and provides a mode of defense not previously described for this pathogen. Staphylococcal protein A (SpA) is the prototypic staphylococcal immunoglobulin binding protein. It is a surface bound protein that binds predominantly to the Fc region of IgG through its repeat domains. This direct coating of the bacteria in IgG is thought to prevent IgG function by orientating the Fc in such a manner so that it cannot activate Fc $\gamma$ RI or complement. In contrast to SpA, SET1 is a secreted protein apparently without multiple repeated binding domains that selectively targets an isotype not previously considered important in defense against staphylococci.

- 10 The ability of SET1 to block IgA binding to its Fc $\alpha$ RI (CD89) suggests a possible role in inhibiting Fc receptor-mediated leukocyte activation that leads to respiratory burst, cytokine release and phagocytosis. The human pathogens *Streptococcus pyogenes* and group B streptococcus also produce IgA binding proteins suggesting there is a selective advantage for these microbes to evade IgA-mediated defense mechanisms. These
- 15 proteins, Arp4 and Sir22, are M proteins from *S. pyogenes* and the unrelated  $\beta$  protein from group B streptococcus all bind IgA-Fc at the same site as the leukocyte receptor Fc $\alpha$ RI, and like SET1, also block IgA binding to Fc $\alpha$ RI bearing granulocytes (26).

The binding and inhibition of complement C5 by SET1 is novel. The site of binding on C5 remains to be identified, but one obvious site would be the C3-convertase cleavage site preventing the formation of C5a anaphylatoxin and C5b initiator of MAC (preliminary evidence of binding of SET1 to activated C5 suggests however that it binds to the C5 beta chain – not shown). Here, SET1 was shown to inhibit complement-mediated hemolysis at concentrations similar to the concentration of C5 in serum, which depends on the formation of the MAC complex and also to inhibit the ability of plasma

25 to effectively kill the gram negative organism *E. coli* at plasma concentration similar to the approximate plasma concentration of C5. The inhibition of complement C5 cleavage to C5a anaphylatoxin would be a more important mechanism to the survival of *S. aureus*. C5a initiates inflammatory responses, leukocyte recruitment and phagocytosis, which are all important for the destruction of gram-positive organisms.

SET1 enhanced microbial survival, inhibiting plasma killing of *E. coli*, an organism that is sensitive to human plasma. *E. coli* was used instead of *S. aureus*, because the latter organism is likely to produce its own endogenous SET1 and is thus capable of better surviving in plasma without additional SET1. Inhibitory activity of this cytolytic  
5 activity was first observed at concentrations above 0.3nM and increased to a maximum of approximately 1.7nM. This concentration is most consistent with the approximated concentration of C5 in serum (0.4nM). Inhibition was not profound with only a maximum of 0.03% surviving colonies (3000 colonies from an initial  $10^7$  inoculum). This would suggest *E. coli* is killed by a number of mechanisms, only one of which (C5  
10 mediated complement) is inhibited by SET1. Notably, the plasma used was not depleted of phagocytic cells that might represent an additional killing mechanism of *E. coli* not inhibited by SET1. SET1 likely represents only a component of a defense mechanism involving multiple factors including other members of the SET (or SSL) cluster. Nevertheless, these preliminary data clearly reveal that SET1 inhibits plasma  
15 bactericidal activity.

Like the superantigens to which they are related, the SETs possess two domains that may also be capable of broad binding capacities. While there are remarkable similarities in the structures of the SETs and superantigens, their functions clearly are very different. Each strain of *S. aureus* carries just one or a few (or sometimes none at all) superantigen  
20 genes. In contrast every strain of *S. aureus* so far examined carries a cluster of many SET genes (3) that indicates first, that this family of proteins is important to the survival of the bacteria and second, that in contrast to the superantigen family that carry out a singular mission of initiating antigen independent T cell activation and cytokine release, the SETs as a family appear to have distinct non-redundant functions. A number of  
25 other recombinant SETs have been tested for IgA binding and/or complement inhibition but so far, only SET1 displays this capacity. Further investigation of other SETs will likely reveal complementary functions involved in pathogen defense.

As an additional bonus, SET1 affinity chromatography offers a simple single step method for the purification of both IgA and C5, which have both traditionally been  
30 laborious molecules to isolate using classical methods.

The invention has been described herein with reference to certain preferred embodiments, in order to enable the reader to practice the invention without undue experimentation. Those skilled in the art will appreciate that the invention is susceptible to variations and modifications other than those specifically described. It is to be  
5 understood that the invention includes all such variations and modifications. Furthermore, titles, headings, or the like are provided to enhance the reader's comprehension of this document, and should not be read as limiting the scope of the present invention.

The entire disclosures of all applications, patents and publications, cited above and  
10 below, if any, are hereby incorporated by reference.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment, or any form of suggestion, that that prior art forms part of the common general knowledge in the field of endeavour to which the invention relates in any country.

15 Throughout this specification, and any claims which follow, unless the context requires otherwise, the words "comprise", "comprising" and the like, are to be construed in an inclusive sense as opposed to an exclusive sense, that is to say, in the sense of "including, but not limited to".

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DATED this 24<sup>th</sup> Day of March 2004  
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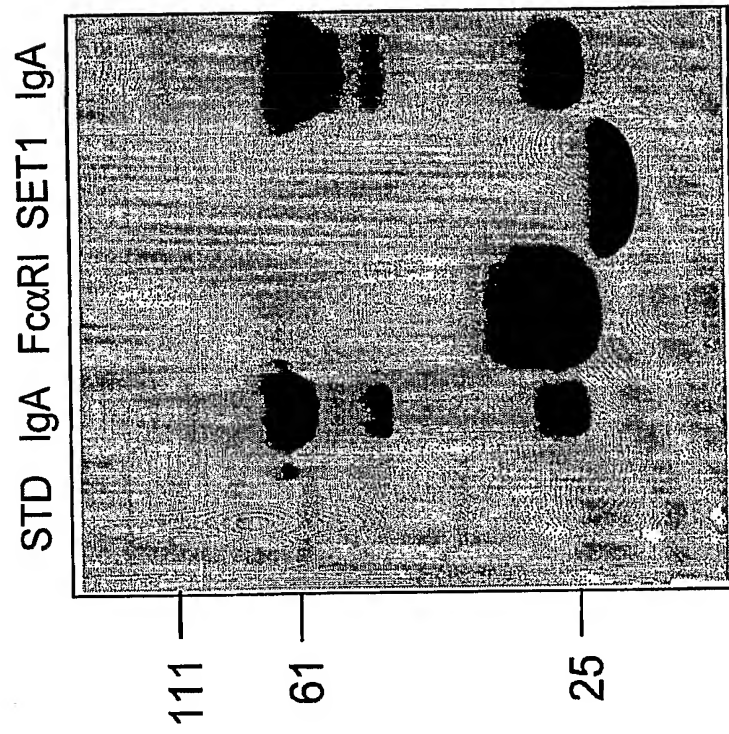


Figure 1

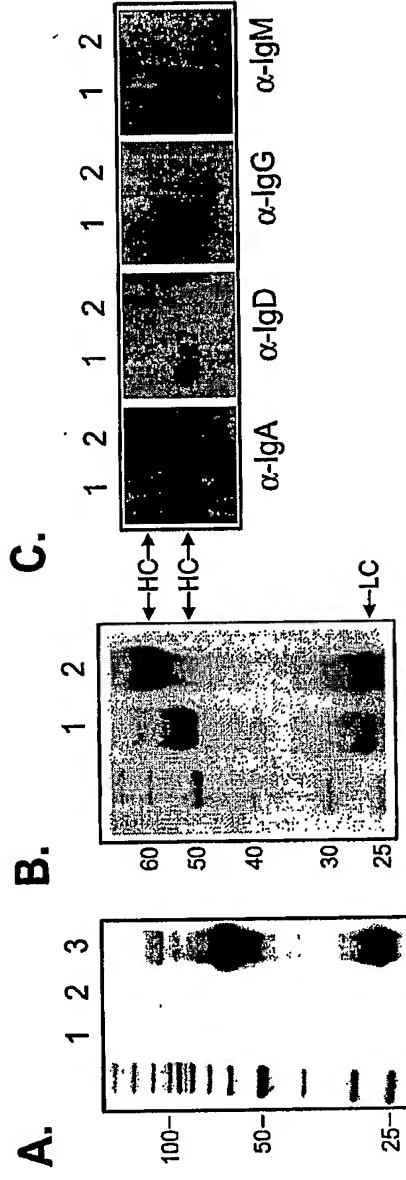


Figure 2



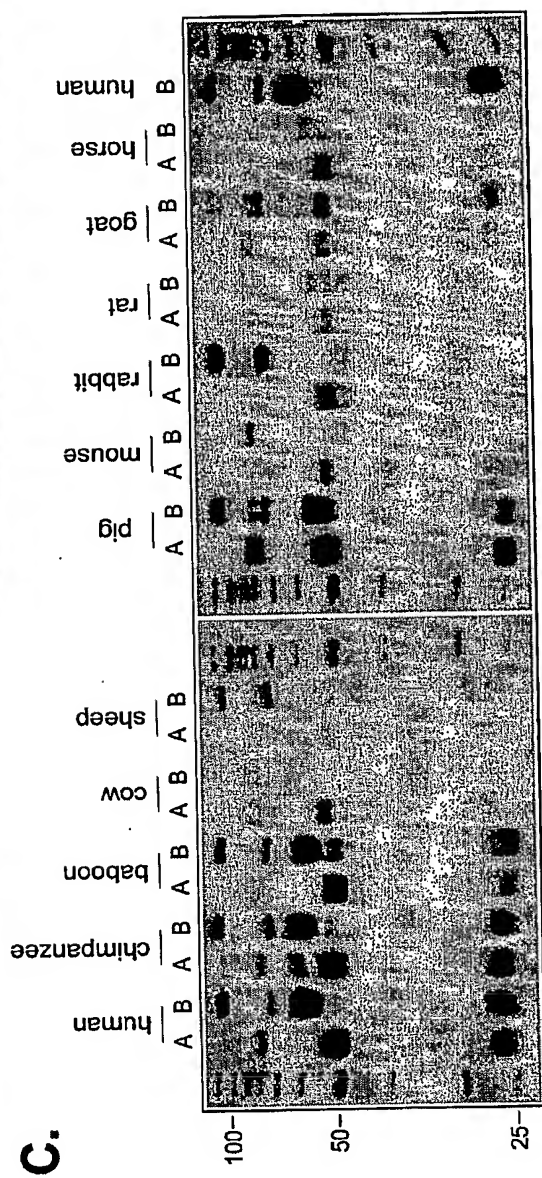
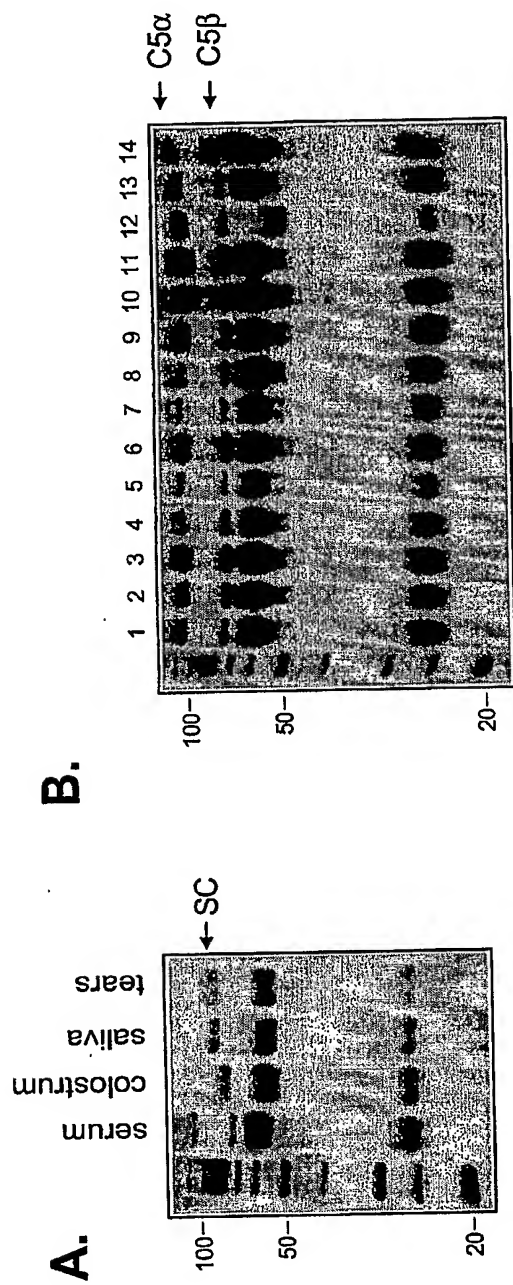


Figure 3

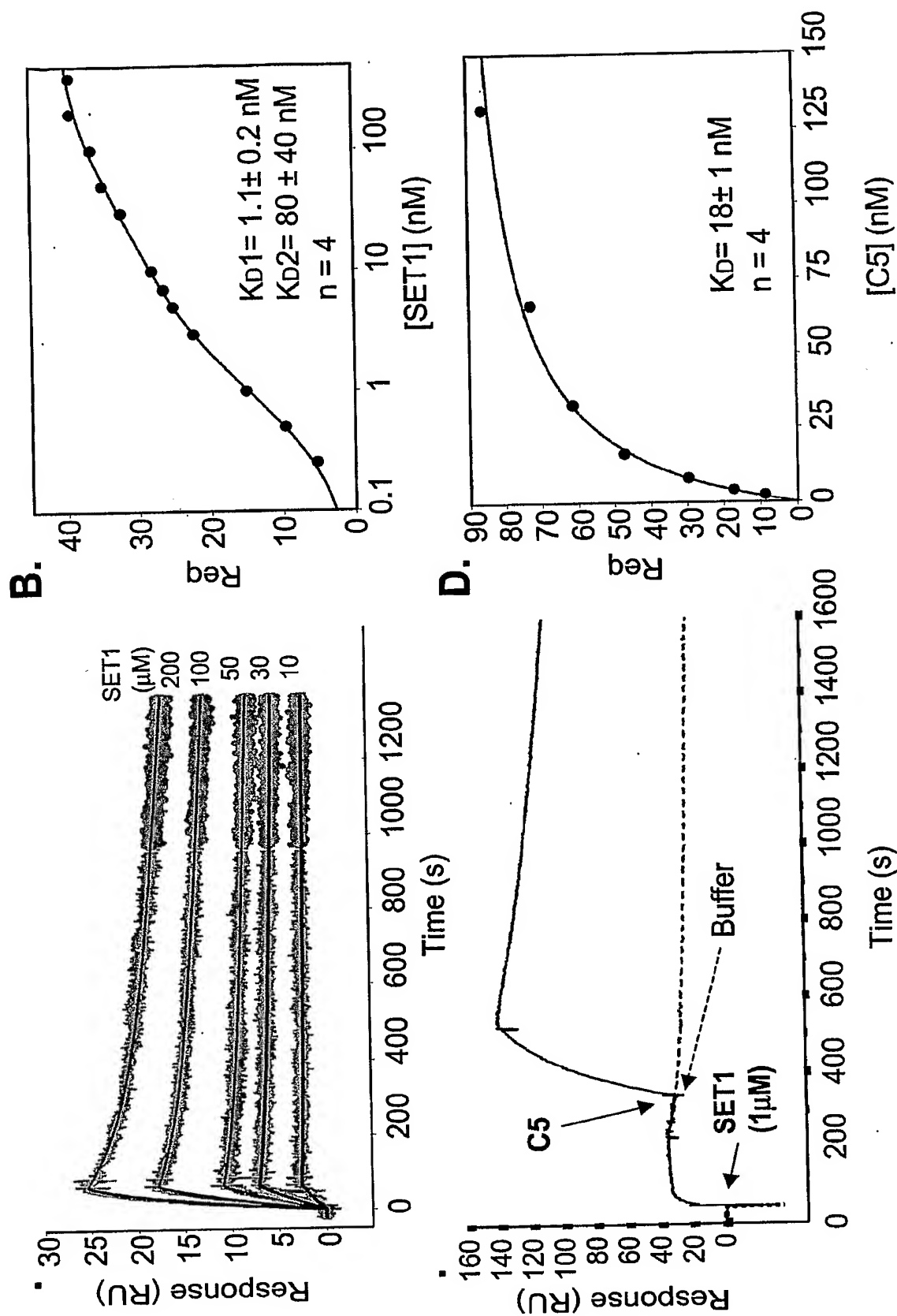


Figure 4

SET1\_GL10  
SET22\_MW2  
SET1\_NCTC8325  
SET1\_GL1  
SET11\_Mu50  
SET11\_N315  
SET1\_NCTC6571

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KEKQERVQHLHYDIKDLHRYYSSESFDFSNISGKVENYNGSNVVRFNQDQGNHQHQLFLLGED
KEKQERVQHLHYDIKDLHRYYSSESPEFSNISGKVENYNGSNVVRFNQENQNHQLFLSGKD
KEKQERVQHLHYDIKDLHRYYSSESPEFSNISGKVENYNGSNVVRFNQENQNHQLFLLGKD
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KEKQERVQHLHYDIKDLRYYSSESPEFSNISGKVENYNGSNVVRFNQEQKNHQHQLFLLGED
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AEKQERVQHLHLDRLHRYYSSESPEYSNVSGKVENYNGSNVVRFNPKDQNHQLFLLGKD
*****:*:*:*****:~:~:*****:*****:*****:~*

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SET1\_GL10  
SET22\_MW2  
SET1\_NCTC8325  
SET1\_GL1  
SET11\_Mu50  
SET11\_N315  
SET1\_NCTC6571

KAKYKQGLEGQNVFVVKELIDPNGRSLSTVGGVTKKNQSSSETNTPLFVKVKVYGGNLDASI  
KDKYKEGLEGQNVFVVKELIDPNGRSLSTVGGVTKKNQSSSETNTPLFVKVKVYGGNLDASI  
KEKYKEIGBKDFVVKELIDPNGRSLSTVGGVTKKNKSSSETNTHLFFVNKVKYGGNLDASI  
KAKYKQGLQGQDFVVKELIDPNGRSLSTVGGVTKKNQSSSETNTHLLVNKLDGGNLDATN  
KDKYKKGLEGQNVFVVKELIDPNGRSLSTVGGVTKKNKSSSETNTHLFFVNKVKYGGNLDASI  
KDKYKKGLEGQNVFVVKELIDPNGRSLSTVGGVTKKNKSSSETNTHLFFVNKVKYGGNLDASI  
KEQYKEGLEGQNVFVVKELIDPNGRSLSTVGGVTKKNKSTSETNTPLFVNKVNGBEDLDASI  
\* . \* \* \* . \* : \* \* \* \* . \* \* \* \* \* \* \* \* \* \* \* \* \* \* \* : \* \* \* \* . \* : \* : \* \* \* :

SET1\_GL10  
SET22\_MW2  
SET1\_NCTC8325  
SET1\_GL1  
SET11\_Mu50  
SET11\_N315  
SET1\_NCTC6571

```

ESFSINKEEVSLKELDPKIRQHLVKNYGLYKGTTKYKGKITFNLKDGEKKIEIDLGDKLQFE
ESFLINKEEVSLKELDPKIRQHLVKNYGLYKGTTKYKGKITFNLKDGEKKIEIDLGDKLQFE
DSFSINKEEVSLKELDPKIRQHLVKNYGLYKGTTKYKGKITINLKDEKQEBIDLGDKLQFE
DSFLINKEEVSLKELDPKIRQVLBEKYGLYQGSTKYKGKITIILNGSKQEBIDLGDKLQFE
DSFLINKEEVSLKELDPKIRQVLBEKYGLYKGTTKYKGKITINLKDEKKVEIDLGDKLQFE
DSFLINKEEVSLKELDPKIRQVLBEKYGLYKGTTKYKGKITINLKDEKKVEIDLGDKLQFE
DSFLINKEEVSLKELDPKIRQVLVNNYGLYKGTSYKGKITINLKDENKVEIDLGDKLQFE
*.*.*.*.*:*****:*****:*****:*****:*.:.*.*****

```

SET1\_GL10  
SET22\_MW2  
SET1\_NCTC8325  
SET1\_GL1  
SET11\_Mu50  
SET11\_N315  
SET1\_NCTC6571

```

HMGDVLNSKDIQNIATVTLKQI
HMGDVLNSKDIQNIATVTINQI
RMGDVLNSKDIQNIATVTLKQI
RMGDVLNSKDIQNIATVTLKQI
RMGDVLNSKDIQNIATVTINQI
RMGDVLNSKDIQNIATVTINQI
RMGDVLNSKDIQNIATVTINQI
RMGDVLNSKDIQNIATVTINQI
*****      ****:***

```

SpA-sepharose + person 1	SET1(GL1) -sepharose	SET1(GL10) -sepharose
person 1		
person 2		
person 3		
no serum		

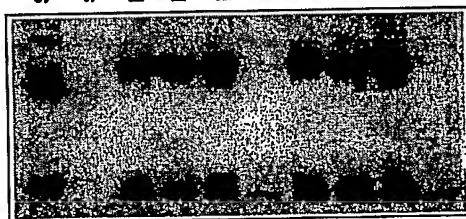


Figure 5

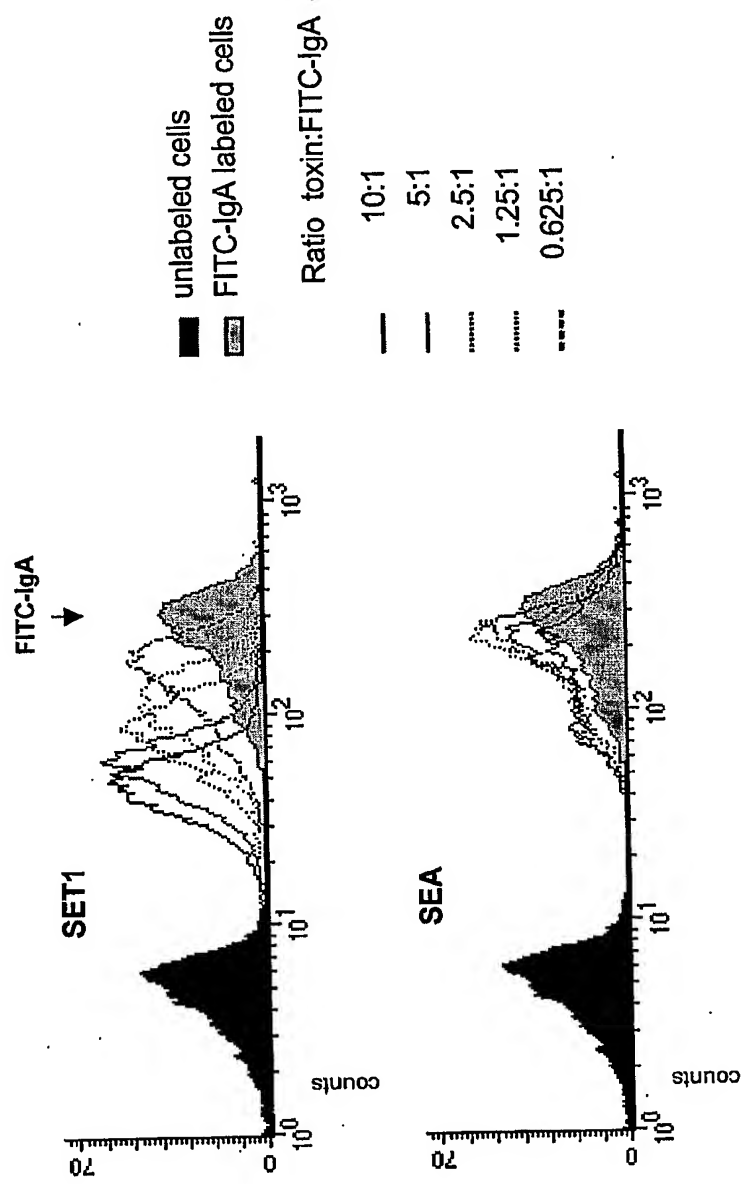


Figure 6

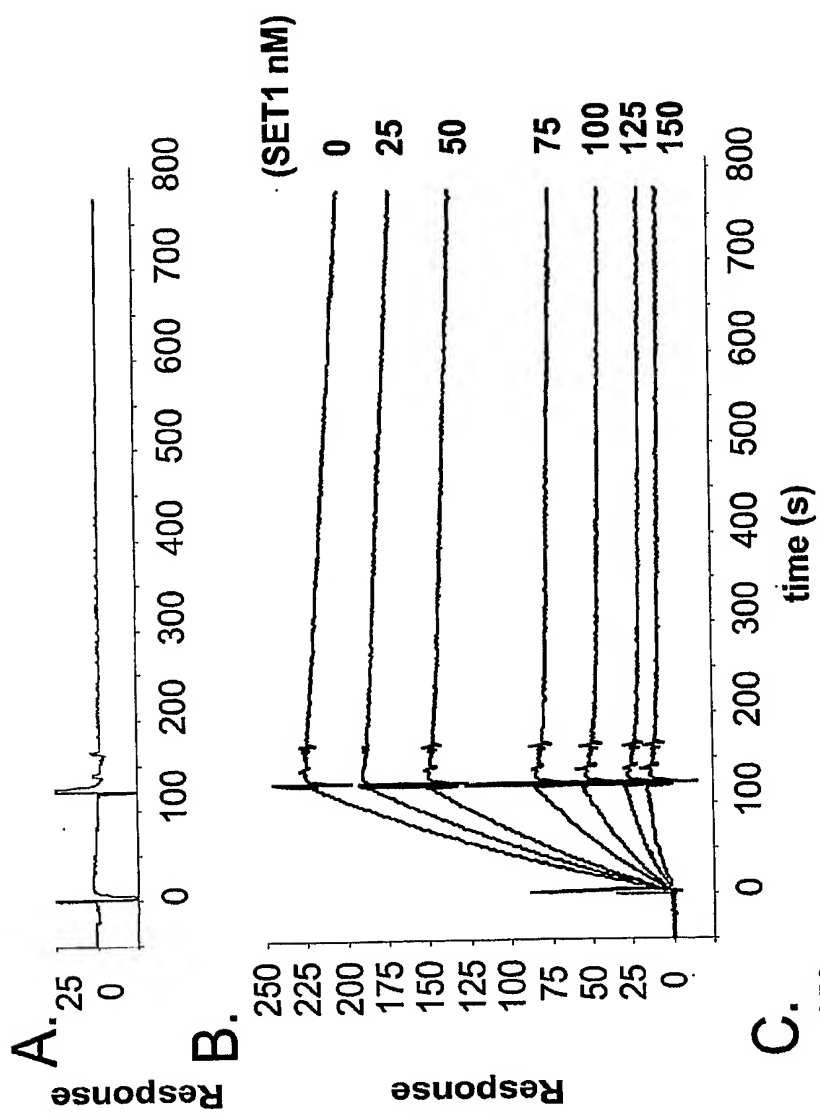


Figure 7

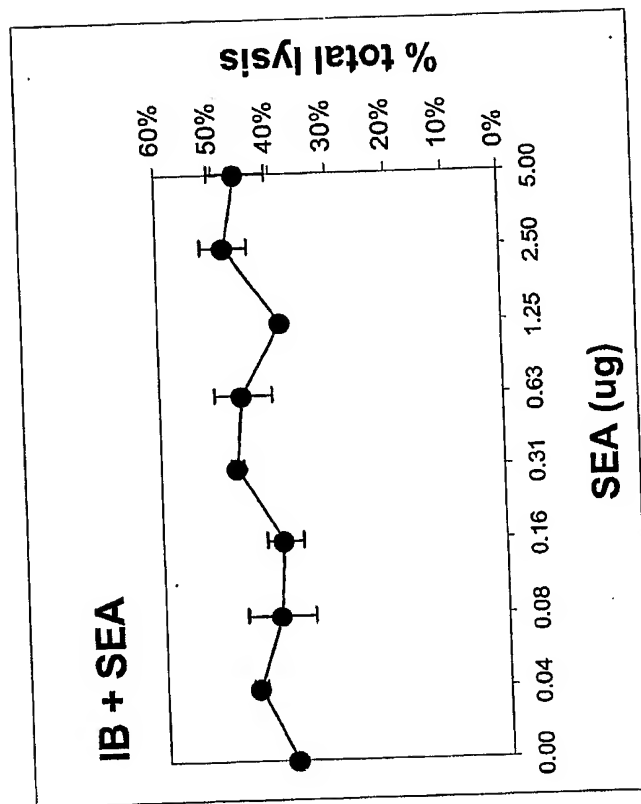
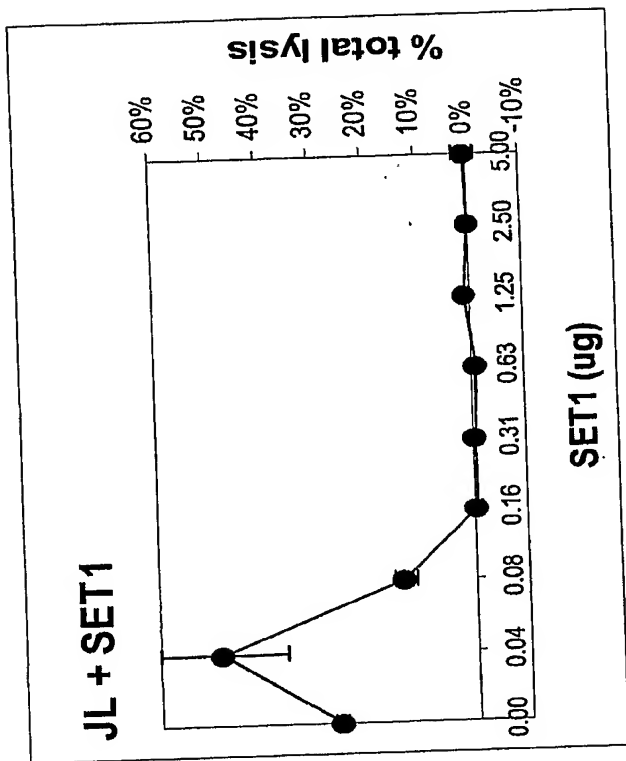
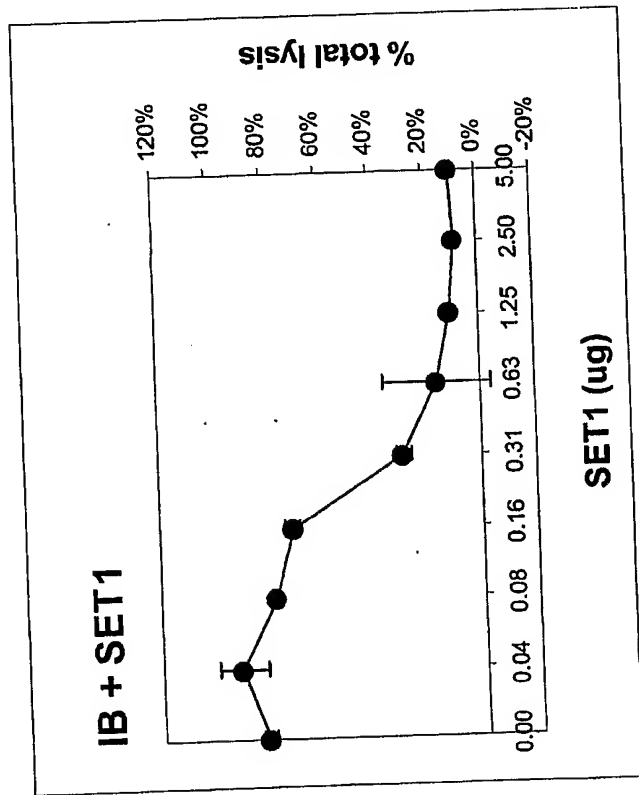


Figure 8

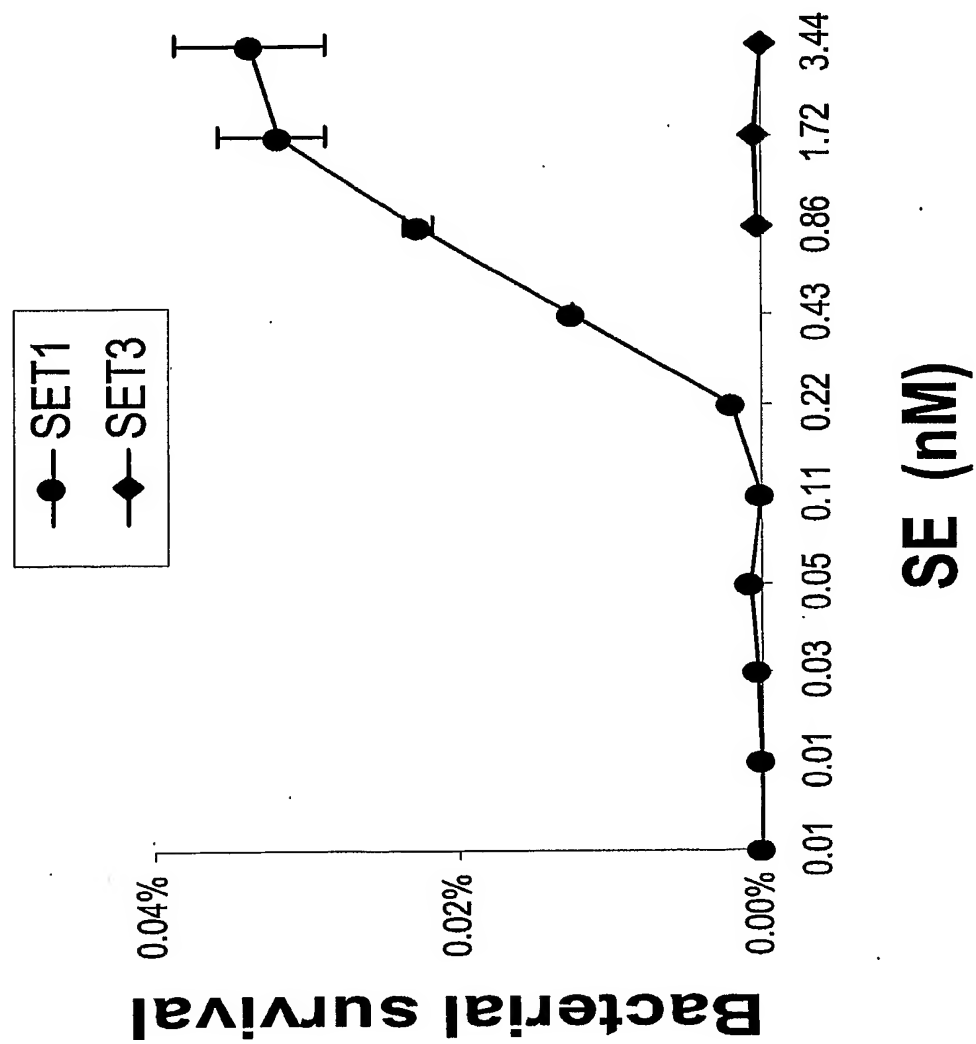


Figure 9